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The development and evaluation of an HPLC method of analysis for nicotine and its major metabolites in urine

THE DEVELOPMENT AND EVALUATION OF AN HPLC METHOD OF ANALYSIS FOR NICOTINE AND ITS MAJOR METABOLITES IN URINE

by

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ABSTRACT

This dissertation details the development and evaluation of an HPLC method of analysis for nicotine and the metabolites cotinine, nicotine-1'-N-oxide and 3' hydroxycotinine in urine samples.

The significance of nicotine, its absorption, metabolism and excretion in man and other animals have been described in Chapter 1.

Chapter 2 deals with the development of the HPLC method of analysis using both isocratic and gradient elution with UV detection. A selection of packing materials/mobile phases covering different retention mechanisms was investigated. A separation of nicotine, cotinine, nicotine-1'-N-oxide and 3' hydroxycotinine and two chromatographic standards, N' acetyl nornicotine and 2-methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine was achieved on a Resolve C_{18} 5 μ radially packed cartridge using gradient elution under reverse phase partition conditions. N' acetyl nornicotine was later discarded in favour of 2-methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine which could be used as an internal standard.

The statistical analysis of the instrument response to nicotine and its metabolites in standard solutions was examined in Chapter 3. A comparison of the measurement parameters peak height and peak area was made. Within-run and between-run precision were calculated. Calibration curves were constructed with Working-Hotelling 95% confidence bands and 95% confidence bounds for 90% of future observations. The limit of detection values were also statistically calculated. Precision was found to be low for some of the components and this was reflected in unacceptably high values of the limit of detection.

The clean-up of urine samples and the extraction of the components of interest were investigated in Chapter 4. Clean-up and extraction proved to be very difficult and analyses of smokers' urine samples underlined the need for an effective clean-up procedure, efficient chromatography and a sensitive and selective method of detection.

It was concluded that the developed HPLC method of analysis was inadequate for quantitative analysis of nicotine and its metabolites in urine.

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I will always remember many 'good times' in Bristol with colleagues past and present from W503, and I thank them for a very happy stay.

Finally, thanks to Mrs. Sue Cottrell, my typist, for bearing with me to the end.

To Mum and Dad

MEMORANDUM

The work described in this dissertation was carried out in the Department of Inorganic Chemistry at the University of Bristol, under the supervision of Dr. M. Cooke, between November 1984 and July 1987. The observations and recommendations herein are those of the author except where reference has been made. No part of this work has been submitted for a degree to this or any other university.

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SYMBOLS

Chromatography

α	relative retention
C_S	concentration of a component in the stationary phase
C_M	concentration of a component in the mobile phase
d_f	effective thickness of stationary phase
D_M	diffusion coefficient in the mobile phase
d_p	particle diameter
D_S	diffusion coefficient in the stationary phase
F	volumetric flow rate in ml sec^{-1}
H	height equivalent to a theoretical plate
ΔH	enthalpy change
k'	partition ratio
L	column length
N	the number of theoretical plates
N_{eff}	effective plate number = L/H
R	gas constant (molar) 8.31441(26) J mol^{-1} K^{-1}
R_s	resolution
T	temperature
t	time
t_M	retention time of an 'unretained' component
t_R	retention time
t_R'	= $t_R - t_M$
u	average mobile phase velocity
V_M	retention volume of an 'unretained' component, also known as the column 'dead volume'
V_R	retention volume

V_R'	$= V_R - V_M$
V_S	volume of the stationary phase
W_b	peak width at the base
$W_{0.5}, W_{\frac{1}{2}}$	peak width at half peak height
γ	obstructive (or tortuosity) factor
Δ	(prefix) symbol for finite charge
λ	column packing uniformity; wavelength
$\sim$	approximately

Statistical analysis

a	the intercept on the y-axis
α	probability level
b	the gradient of the regression line
F	Snedecor's F statistic (Tables)
n	no. of trials or observations
Q	Dixon's Q, used to test for outliers (Tables)
r	correlation coefficient
s	standard deviation
s^2	variance
s_B	standard deviation of the blank
s_{YX}	standard error
t	student's t statistic (Tables)
V_i	injection volume
x	$X-\bar{X}$
$\bar{X}$	mean value of $X = X_i/n$
Σx^2	$\Sigma(X-\bar{X})^2$
X_{LD}	statistical limit of detection based on its calibration data (99% confidence)

x_p	a selected value of X
y	$Y-\bar{Y}$
$\bar{Y}$	mean value of $Y = \Sigma Y_i/n$
Y	predicted value of the true response for a given value of X
y_B	blank signal
Y_L	the lower limit on that predicted individual X value which exceeds the 99% upper prediction limit on the expected blank, Y_{UB}
Y_Q	the response calculated from the regression line which corresponds to X_{LD}
Y_{UV}	the 99% upper prediction limit on the expected blank value, a
z_p	the normal deviate (Tables)
ν	degrees of freedom
Σ	summation of
χ^2	chi-squared statistic (Tables)

ABBREVIATIONS

AFID	alkali flame ionization detector
a.u.f.s	absorbance units full scale
CNS	central nervous system
CO	carbon monoxide
CO_2	carbon dioxide
CoHb	carboxyhaemoglobin
COT	cotinine
ED	electrochemical detection
Et_3N	triethylamine
Et_2NH	diethylamine
FID	flame ionization detector
f.s.d.	full scale deflection
FTIR	Fourier transform infra-red detection
GC	gas chromatography
HCN	hydrogen cyanide
HPLC	high performance liquid chromatography
3HC	3' hydroxycotinine
IC	ion chromatography
ID	internal diameter
IIA	ion-interaction agent
IIC, -IIC	ion-interaction chromatography
IPA	ion-pairing agent
IR	infra-red
IS	internal standard
i.v.	intravenous

LC	liquid chromatography
LOD	limit of detection
MeCN	acetonitrile
MeOH	methanol
MS, -MS	mass spectrometry
mV	millivolt(s)
N.	Nicotiana
NANN	N' acetyl nornicotine
NEN	N ethyl nornicotine
NIC	nicotine
NNO	nicotine-1'-N-oxide
ODS	octadecylsilane
PDFOA	pentadecafluoro octanoic acid
RCM-100	Radial Compression Module - 100
RI	refractive index
RIA	radioimmunoassay
RP	reverse phase
RSD	relative standard deviation
SD	standard deviation
TLC	thin layer chromatography
UV	ultra-violet detection
vs.	versus
~	approximately

CHAPTER 1

GENERAL INTRODUCTION

1.1 History of Smoking

Tobacco smoking was first introduced to Europe in the form of pipe smoking by the Spanish explorers in the early 16th century. Explorers of the New World brought it to England and by 1590 sufficient quantities were being imported for Queen Elizabeth I to impose the first import duty of 2d. a pound.[1]

Tobacco was initially introduced into several countries for its medicinal value. Jean Nicot, French Ambassador to Portugal from 1559-1561, after whom nicotine was named, informed the Queen of France, Catherine de Medici, that tobacco smoke led to "a quiet tranquility and great submissiveness of disposition, so that through general use of tobacco, Her Majesty's subjects would become easy to govern". A number of writers since that time have also characterised tobacco as having tranquilizing properties.[2] During the Great Plague in London in 1665, children were told to smoke in their classrooms and grave-diggers who used tobacco as a deodorizer had unwittingly protected themselves against infection.[3]

Tobacco has always been the subject of acute controversy which continues to the present day. It has not only been praised as a preventive against many ills but it has been condemned as a noxious vice, in particular by James I in his "Counterblaste to Tobacco". The British Medical Journal also took up the fight as early as 1889 when its editorial stated, "we advise non-smokers not to put their trust in pyridine during the prevalence of fevers and to remember that their tobacco-loving friends owe their immunity to good health and strength, which enables them to stand tobacco, and, at the same time, to resist infection".[4]

Throughout the 17th century, tobacco consumption in England rose steadily, mostly in the form of pipe smoking, but it was also chewed and snuffed. Snuff-taking became very popular with the aristocracy towards the end of the 17th century. Cigarettes were first made in Spain in the mid-17th century. This form of smoking may have been introduced into England by troops returning from the Crimean War. Cigarettes only began to be popular at the beginning of the present century, since then they have steadily tended to replace other forms of smoking in Britain.[1]

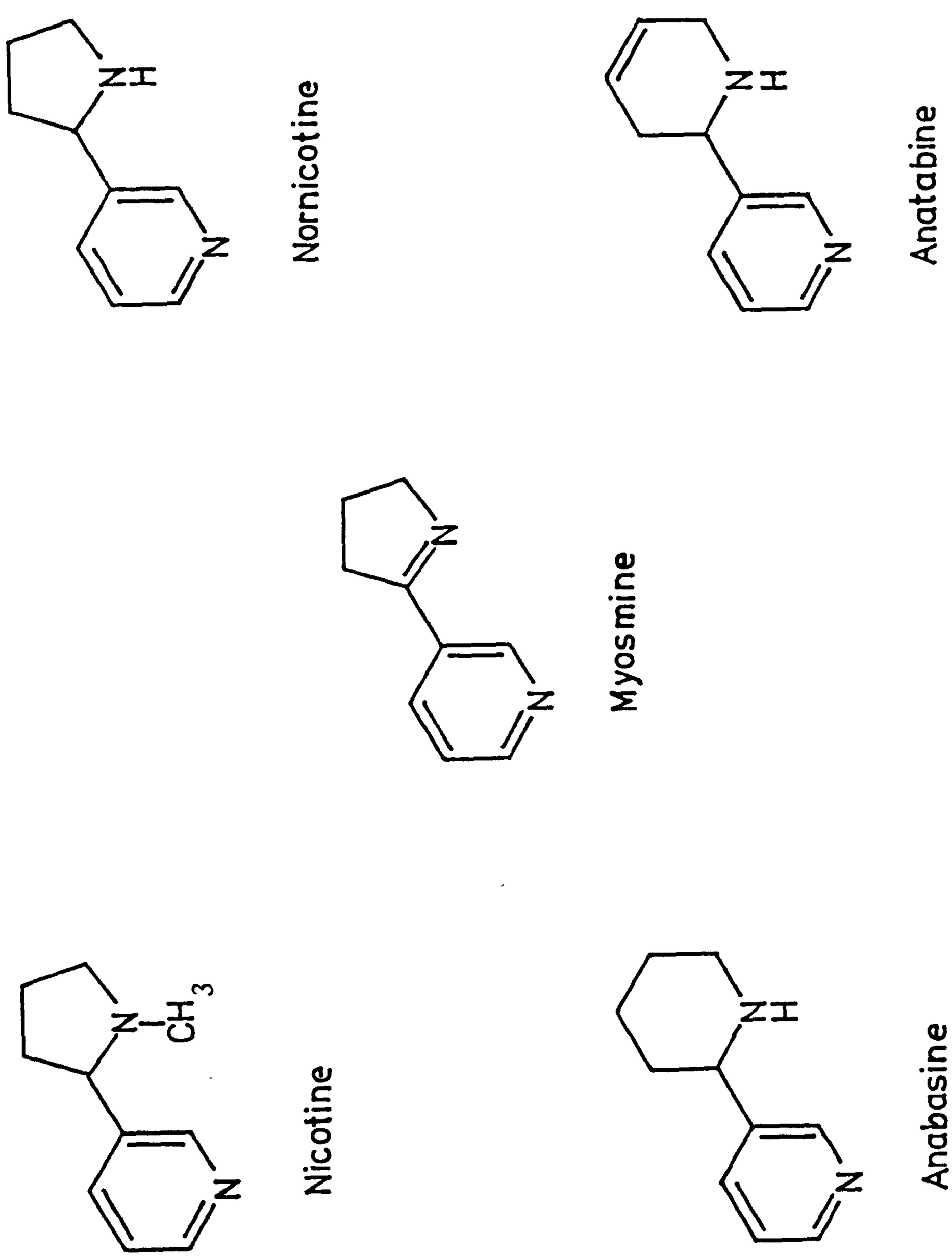
1.2 The Tobacco Plant

Alkaloids are plant substances of basic reaction containing nitrogen and have characteristic effects on animal organisms. Chemical similarities between them are limited to their basic structure which includes various heterocyclic rings.

Nicotine is the primary alkaloid in Nicotiana tabacum and Nicotiana rustica and although it is by far the most important, it is not the only one found. The minor alkaloids most commonly found include nornicotine, anabasine, anatabine, myosmine and trace amounts of some others of the pyridine class (see figure 1.1).

Tobacco grows like any other plant, but it has an unusally large leaf area. The tobacco plant has the ability to synthesize and accumulate the alkaloid nicotine, the majority of which (∿ 90%) is produced in the roots and transported to the leaves for storage. Harvesting is concerned only with the composition of the leaves and

Figure 1.1: Tobacco Alkaloids



perhaps the stalk and a small number of seeds for reproduction.

There are more than 60 genetic species of plants belonging to the genus Nicotiana. Only two of them, N. tabacum and N. rustica, are cultivated for use as tobacco. Dawson[5] has suggested that their survival was due only to man's interference owing to their uniquely high proportion of alkaloids (nicotine) and the fact that native tobacco use was almost entirely confined to those species which contained nicotine. N. tabacum is grown extensively in many (∿ 100) countries throughout the world and constitutes the tobacco of commerce. The popular assumption is that inhalation of tobacco smoke is the sole source of this alkaloid. However, small quantities of nicotine can also be found in tomatoes, peppers and eggplants.[6]

1.3 Treatment of the Tobacco Plant before use: Curing, Fermentation and Additives[7]

Nicotine may form either neutral or acidic salts. The form of occurrence in tobacco is important to the smoking properties. In green tobacco it is mainly present as free nicotine, but a varying proportion may be in the form of salts in the cured leaf, according to the type of curing.

Most tobaccos are processed by an initial stage called curing. The mature green leaf of the tobacco plant after harvesting is subjected to particular conditions of humidity and temperature. Curing is basically an imperfectly controlled autolysis. There are essentially four kinds of curing processes, namely, flue-curing (heat-curing),

air-curing (natural curing), fire curing and sun-curing. Strictly speaking, each process requires air i.e. none is anaerobic.

The process of flue-curing concerns the major part of the world's tobacco production. It takes place in tightly constructed barns that are heated by furnaces, the entire process requiring only 5-7 days. This produces a tobacco giving an acidic smoke of light aroma in contrast to the product of air-curing with its alkaline smoke of fuller aroma. Air-curing is a procedure characterized by slow, gradual drying of the leaf, requiring 30-40 days for completion. Sometimes charcoal fires must be employed to prevent moulding and spoilage. Cigar tobacco and also some dark types are air-cured. Oriental tobaccos are sun-cured, cured by exposure to sunshine and are mostly grown in countries bordering the Aegean, Mediterranean and Black Seas. Dark fire-cured tobaccos are hung in closed barns and permitted to absorb products from open hardwood fires. Leaves cured in this manner are not used in cigarette blends, but are employed mostly in the manufacture of snuff (powdered tobacco).

Little nicotine (0.1%) is lost during curing but in all cigar and some oriental tobacco types curing is followed by another operation called fermentation. Until the cured leaf of cigar tobacco has been fermented, it is totally unfit for cigar manufacture. Fermentation (sweating) is a complicated procedure, during which both physical and chemical properties of the leaf are drastically altered. Considerable nicotine may be converted to partial degradation products including nicotine 1'-N-oxide, 3-pyridylmethylketone, 3-pyridylpropyl-ketone, N-methylnicotinamide, nicotinamide, myosmine, cotinine and nicotinic acid. Although very little nicotine is lost during curing

it is not merely a process of drying or desiccation. Many enzymes within the leaves produce changes in the organic substances, especially the carbohydrates and proteins. Further changes occur during storage and fermentation, all of which are intended to improve the taste and smell of the product. Pyridine derivatives produced during these various processes will volatilize with nicotine and its combustion products so contributing, at least qualitatively, to the chemistry of the smoke produced.[8]

The composition of the leaf is not only affected by curing. Climatic factors, additives and the paper used all contribute to the make up of the smoke. Climatic and mineral-nutrient factors modify the physical and chemical characteristics drastically, as shown by the fact that leaves of different composition, texture and quality are obtained from the same lot of seeds when they are planted in different parts of the world.

"Saucing" materials or "casing" solutions containing licorice, sugars, syrups, honey, chocolate, balsams and other flavouring materials may be added to the leaf before cutting and alcohol-soluble flavours such as fruit extractives, menthol, oil of peppermint, oil of cloves, oil of cinnamon or other aromatic substances may be applied after cutting. These materials are added to improve the flavour, aroma and smoke taste. Characteristics of the smoke components are modified by these additives. Some pipe tobaccos may contain up to 30% saucing and casing material, a factor that appears to influence the concentration of some smoke components.

1.4 Cigarette paper

Cigarette paper is manufactured from flax fibre. To regulate its porosity and burning characteristics and to increase its opacity, the paper is impregnated with 13-20% calcium carbonate and other salts, usually citrates or phosphates. In some parts of the world, rice paper, dried young banana leaves, parchment paper, or tobacco leaf itself are employed as cigarette wrappers.

Differences in the make up of tobacco and all the above mentioned factors affect the smoke constituents resulting from their combustion. Thus, a knowledge of tobacco products obviously contributes to an understanding of smoke components which are derived from these materials.

1.5 Tobacco Smoke[7,9,10]

Smoke formation in any burning process is due to incomplete combustion. Carbon dioxide and water are the main products of complete combustion. In tobacco smoking, several factors contribute to the formation of smoke, such as poor combustibility of certain leaf constituents, insufficient supply of oxygen and the existence of a temperature gradient in the hot zone.

During the combustion process at least three types of reactions occur simultaneously: pyrolysis, pyrosynthesis and distillation. During tobacco burning, thermal decomposition (pyrolysis) occurs where the organic matter is broken up into small fragments. The newly formed fragments being partially unstable can recombine to

form components not originally present in the tobacco. This step is called pyrosynthesis. The third process known to occur is the distillation of certain compounds such as nicotine and some terpenes. That tobacco smoking is so popular is thought to be due to the fact part of the nicotine contained in tobacco is transferred to the smoke in unchanged form by this distillation process.

The smoke is an amalgam effect of the tobacco blend, its rate and form of combustion, the paper and the action of the filter. The tobacco blend is not merely the sum of its components. Not all tobaccos 'marry' together to give a satisfactory blend.

Over 2,000 components have already been identified in smoke and it is expected that many more remain to be discovered. The very complex nature of cigarette smoke is studied in two fractions: the particulate phase (total particulate matter (TPM) or, erroneously, tar) and the gaseous (vapour) phase. The TPM not only contains the non-volatile particulates but also the semi-volatiles like nicotine, linoleic acid, palmitic acid, cresol, glycerol and phenol. In general it is considered to be composed of three main parts: water (∿ 10%); tobacco alkaloids (∿ 10%) and tar (∿ 80%).[11] The gaseous phase, although it amounts to less than 2% by weight of the total emission, contains many important compounds including the combustion products like CO and CO_2 and also those irritants most noxious to the non-smoker such as aldehydes and oxides of nitrogen. Lists of some important components in the gas and particulate phases are presented in tables 1.1 and 1.2.

TABLE 1.1: Cigarette smoke: gas phase components (µg/cigarette)[182]

Carbon monoxide	13,400
Carbon dioxide	50,600
Ammonia	80
Hydrogen cyanide (hydrocyanic acid)	240
Isoprene (2-Me-1,3 butadiene)	582
Acetaldehyde	770
Acrolein (2-propenal)	84
Toluene	108
N-Nitrosodimethylamine	0.08
N-Nitrosomethylethylamine	0.03
Hydrazine	0.03
Nitromethane	0.5
Nitroethane	1.1
Nitrobenzene	25
Acetone	578
Benzene	67

TABLE 1.2: Cigarette Smoke: particulate phase components (µg/cigarette)[182]

Nicotine	1,800
Phenol	86.4
o-Cresol	20.4
m-and p-Cresol	49.5
2,4 Dimethylphenol	9.0
p-Ethylphenol	18.2
β-Naphthylamine	0.028
N-Nitrosonornicotine	0.14
Carbazole	1.0
N-Methylcarbazole	0.23
Indole	14
N-Methylindole	0.42
Benz(a)anthracene	0.044
Benzo(a)pyrene	0.025
Fluorene	0.42

That part of the cigarette smoke which passes through a conventional Cambridge filter is defined as the organic gas phase. The TPM is that portion which is retained on the filter pad.

Tobacco smoke is really a concentrated aerosol containing many semi-liquid particles in a gaseous phase. As many as 10^8-10^9 particles/cm^3 may be present.[12] The total range of particle sizes is approx. < 0.1 μm to 1 μm in diameter and the Cambridge filter will trap 99.9% of those greater than 0.1 μm.

Cigarette smoke can be classified as:

(i) mainstream smoke, which is drawn through the cigarette, by the smoker, during a puff. It is acidic in nature.

(ii) sidestream smoke, the portion which leaves the cigarette from the area around the coal directly into the air. It gives an alkaline reaction[13] (see figure 1.2). The chemical composition of the main-stream smoke is dependent on the temperature profile during the actual puffing, while the yield in the sidestream smoke depends on the smouldering and with it the static burning rate during the puff intermissions. The mainstream smoke is appreciably less than half the smoke volume and both particulate and gaseous phases are derived entirely from approximately the outermost 25% of the cigarette radius. The periphery burns more strongly than the apex and reaches the maximum temperature of 900-1000°C while being puffed whereas the temperature in the centre of the coal is approximately 800°C with a temperature drop of 100-150°C between puffs. The coal temperature has a significant effect on smoke composition. The particulate matter of the sidestream smoke comes mainly from the periphery but its gaseous phase, including most of the water, originates in the slower burning core, particularly between puffs. Although the smoke is formed in an instant of combustion

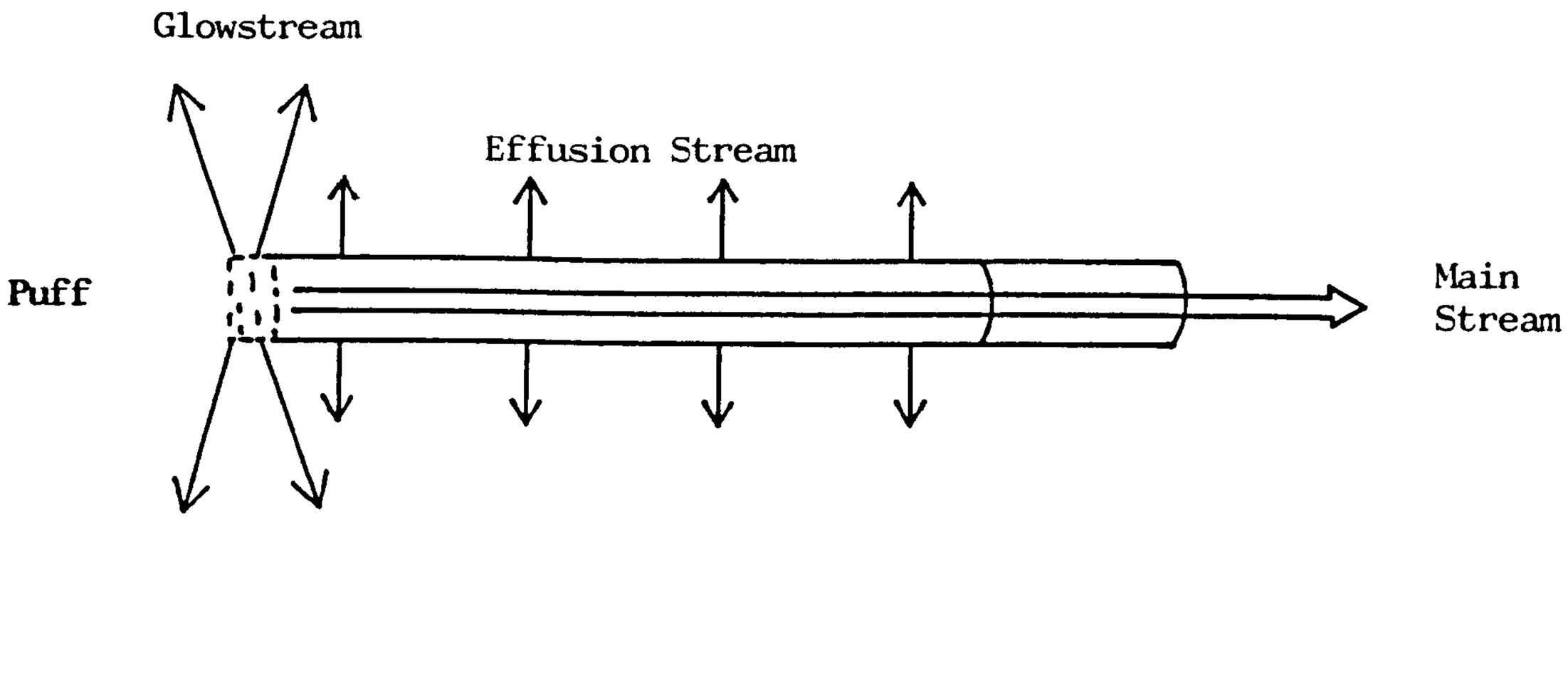


Figure 1.2: Smoke streams of the cigarette[13]

at a very high temperature it passes through a very rapidly falling temperature gradient. The majority of the mainstream smoke reaches the puff end of the cigarette at ambient temperature but for the final puff or two it is likely to be around 50-75°C. The higher temperature alters its ability to distil compounds from the remaining tobacco rod through which it passes. This has been verified by Ashton et al. who showed that less nicotine is available to the smoker in the first half of a cigarette.[14]

The exact composition and amount of smoke produced are not only affected by the amount and type of tobacco used, additives and paper but also by a number of physical variables. These variables include the pressure drop (draw resistance), volume, duration and frequency of puffs, butt length and moisture content and the presence of filters and their efficiencies. In a report by Bradford et al.[15] using a smoking machine to estimate the amount of nicotine entering the mouth, the authors found variations even under constant machine conditions due to moisture content and packing differences.[15] Changing smoking conditions such as puff frequency or volume caused drastic changes in nicotine transfer.[16]

Attempts have been made in many experimental studies involving smokers and in the use of smoking machines which simulate human smoking habits, to standardize these parameters. For example, the cigarettes are conditioned at constant temperature (21°C) and humidity (60-70%) for ∿ 24 hours prior to use,[17] then smoked at the rate of 1 puff per minute of 2 seconds duration and a puff volume of 35 cm^3 leaving a butt length of 23 mm.[18]

1.5.1 Nicotine and other alkaloids - transfer to smoke

The nicotine content of cigarettes has shown a gradual decline in recent years. An average cigarette contains ∿ 10 mg of nicotine. The mainstream smoke which is taken into the smoker's mouth contains between 1 and 2 mg of nicotine per cigarette (for modern filter cigarettes) and of this 1-2 mg it has been found that over 90% is absorbed into the bloodstream via the lungs.[19]

The mainstream smoke from cigarette tobacco shows an acidic

reaction (pH 4.5-6.0) and it has been found that the nicotine from this smoke is best absorbed into the bloodstream if the smoke is taken into the lungs. This is partly due to the vast surface area for absorption and the pH of the alveolar surface fluids is ∿ 7.4 as opposed to 5.5 in the case of cigarette smoke. The pH of saliva is ∿ 5.5.[20] As only ∿ 20% of the nicotine is present in the mainstream smoke, there is therefore a much greater amount of nicotine in the sidestream smoke. Like smoke from pipes and cigar tobacco, sidestream smoke is alkaline (pH 8-9), which produces a strong irritating effect. This is the reason pipe and cigar smoke are rarely inhaled. In alkaline smoke nicotine can readily be absorbed in the mouth. This means that the body can absorb nicotine without inhalation;[21] hence the concept of the "passive" smoker, a person exposed to environmental tobacco smoke. A passive smoker can accumulate nicotine by being present in a smoky atmosphere.

There are a large number of alkaloids and other bases in cigarette smoke but nicotine is possibly the single most characteristic tobacco compound and pharmacologically the most important.

The distribution of nicotine during the smoking of a cigarette was studied by Harlan et al.[22] Under the standard conditions, one puff/minute, 35 cm^3 puff volume and a butt length of 23 mm, the following percentages were obtained;

mainstream smoke	:	20%
sidestream smoke	:	40%
butt	:	6%
destroyed (pyrolyzed)	:	34%

This transfer rate was established with plain cigarettes (without

a filter). The transfer rates have now decreased due to very efficient filters and the use of particularly well-burning tobaccos.

While much work has been done on the quantitative presence of nicotine in tobacco smoke, very little is known about the secondary (minor) alkaloids. As they make up only ∿ 5% of the total alkaloid content of the cigarette, nicotine being the other 95%, they are present in extremely small quantities (microgram and submicrogram levels). These alkaloids, although present in only trace amounts in one cigarette, over a number of years the smoker will be exposed to a considerable quantity.[23] Kuhn[9] has compiled some of the available information on the transfer of minor alkaloids present in tobacco to the smoke. Transfer by direct distillation depends to a greater or lesser extent on thermal stability and volatility. Nornicotine is the quantitatively predominating secondary alkaloid: Jeffrey and Tso[209] reported 1.16% in tobacco. However, nornicotine is pyrolyzed by smoking to a much greater extent than nicotine. Only 4-5% of nornicotine is transferred to the mainstream smoke, ∿ 3% to the side-stream smoke and 1% of the alkaloid remains in the butt. Myosmine, anabasine and anatabine have also been identified in cigarette smoke, their presence being due to direct distillation, however myosmine is also a pyrolysis product of both nicotine and nornicotine. The only other alkaloid found in sizable quantities was cotinine. Nicotine-1'-N-oxide, also found in tobacco, is unable to pass into smoke itself but may decompose to give nicotine and hence pass into the smoke in this form. Kuhn[9] concluded that secondary alkaloids were present in smoke in approximately the same ratio to nicotine as in tobacco.

1.5.2 Pyrolysis and Pyrosynthesis[9]

As approximately 30% of the nicotine and perhaps 90% of the nornicotine is pyrolyzed during smoking, it is expected that pyrolysis products will occur in both mainstream and sidestream smoke. During pyrolysis the pyrrolidine ring of both nicotine and nornicotine is readily attacked and split, with the result that the major heterocyclic smoke constituents deriving from the alkaloids are pyridine and its derivatives. The many reactions which take place, dehydration, dehydrogenation, de-amination and cracking, produce a wide range of products. Nicotine produces a series of simple degradation products; however, identification is still difficult owing to the extremely low concentrations of these compounds formed. Pyrosynthesis will also occur producing new compounds not present in tobacco or present in only small concentrations. An interesting example of pyrosynthesis is the 100-fold increase in the concentrations of harmane and norharmane in going from tobacco to tobacco smoke. Although many of these products may be of qualitative importance in cigarette smoke, in all other respects nicotine is outstanding.

1.6 Toxicity

Nicotine is one of the most toxic of all drugs and acts with a rapidity comparable to that of cyanide. The fatal dose for man is approximately 60 mg, although 4 mg or less usually produce alarming symptoms in non-habitual users.[24] The nicotine content of one cigar may exceed one lethal dose for man, however, as already stated, the absorption of nicotine is dependent on the pH of the smoke. The alkaline reaction of cigar smoke means the actual absorption of nicotine is not strictly related to the quantities of nicotine contained either in the tobacco or in the smoke.

The fact that nicotine output from the body is always less than the nicotine input leads to the conclusion that either nicotine is stored in the body or that it is broken down in some way. Knowing the toxicity of nicotine and the extent of the intake by the smoker makes storage unlikely, so to prevent the accumulation of the absorbed alkaloid it is possible for the body to degrade the nicotine very quickly and to eliminate it. In fact it is for this reason that smoking and the inhaling of nicotine can be practised.

1.7 Nicotine Absorption

Nicotine reaches the brain, in a smoker who inhales, more rapidly than the heroin addict can get a "buzz" when heroin is injected into a vein.[20] Nicotine in acidic smoke absorbed through the lungs reaches the brain more quickly than after an intravenous injection. It takes only 7 seconds for nicotine absorbed through the lungs to reach the brain, compared with 14 seconds required for blood to flow from arm to brain.

It is worth noting that nicotine may not be wholly responsible for the addiction to cigarette smoking. It is almost certain that nicotine is the reason why people smoke, smoking as a habit has never been practised in the absence of a pharmacologically active alkaloid. Although monkeys and rats will learn to self-inject nicotine for its own sake, indicating that it has some action which they find rewarding, it has not been shown that intravenous injection of nicotine alone would be used as a substitute for tobacco although it is extremely difficult to simulate cigarette smoking and the efficiency with which nicotine is administered. It is possible that some other constituent in the smoke, perhaps in the semi-volatile fraction, acts as a reinforcer. Also the 'puff-by-puff' form of inhalation addicts the smoker. This method not only provides reinforcement but a minimal delay time between response and the reinforcement. Furthermore, each inhaled puff produces a 'bolus' of blood containing a high concentration of nicotine, many times higher than the levels shown after mixing or those obtained by slower absorption of much larger quantities of nicotine. A smoker who smokes twenty cigarettes per day, taking ∿ ten puffs per cigarette, will get in excess of 70,000

high nicotine boli (shots) to his or her brain in one year. It is therefore not so surprising that cigarette smoking is highly addictive. In an attempt to simulate this type of intake, investigators have used a series of intravenous injections between 1 and 2 µg/kg equating it with the inhaling smoker who will receive the nicotine intermittently during the 5 or 10 minutes he or she is smoking.[24]

Modern flue-cured cigarettes have encouraged, if not promoted, a switch to this very addictive form of nicotine intake. This milder acidic smoke minimizes the slower absorption through the buccal and nasal mucosae, forcing the smoker to inhale to achieve a pharmacological effect. Once accustomed to the high nicotine peaks obtained on inhalation, smokers inhale even the more irritating alkaline smoke from which nicotine can be absorbed without intake into the lungs, and they are not satisfied by the slower rate of absorption.

1.8 Self-titration and Tar-nicotine Correlation

Smokers literally have "fingertip control" over how much nicotine or other smoke constituents are taken into the body.[25] A simple change in the smoking puff results in a different composition of the smoke. Although nicotine appears to be the major factor controlling smoking behaviour, acting as a pharmacological reinforcer, in commercial cigarettes tar and nicotine content covary ($r = 0.96$).[26] Several studies have shown that the smoker compensates for changes in the nicotine (tar) yield of the cigarette by changing smoking behaviour.[27-31] There is a downward 'titration' when the smoker has

made a switch to high-nicotine (high tar) cigarettes and a limited upward 'titration' when a change to low nicotine (low tar) cigarettes is made. It has been assumed, but not proven, that this self-regulation or 'self-titration' is related to nicotine and not some other covarying constituent. In fact, Gust et al.[17] have found that puff volume, and hence the intake of smoke, was inversely related to the nicotine content of a cigarette even when tar and carbon monoxide levels remained constant, but Robinson et al.[32] also suggest that compensation is almost complete not only for nicotine but also for CO and HCN. Although most studies reported 'self-titration' when comparing high-nicotine yield cigarettes with the smokers' own brand, evidence on nicotine regulation with low nicotine cigarettes was not always found. The main determinant of whether a cigarette has a low or a high nicotine yield in machine testing are the characteristics of its ventilation and burning. Tobacco from low-yield cigarettes does not contain less nicotine, therefore the nicotine/tar is available to the smoker under different smoking conditions. Smokers, in an attempt to keep their intake of nicotine constant, may increase their exposure to other smoke components by smoking the low nicotine/tar cigarettes more intensively or inhaling more deeply, that is, they take in more tar and CO per unit of nicotine. This may partially explain the limited ability of smokers to 'titrate' upwards when the nicotine yield of the cigarette is low.[33] They may have difficulty in tolerating the increased smoke intake that would be required to compensate for the lower delivery of nicotine. Russell et al.[34] speculated that offering ultra-low nicotine cigarettes would make up-regulation very difficult and therefore incomplete. In a study

by Dunn and Freiesleben[35] evidence was found to support the view that lung exposure to smoke decreased when smoking the high nicotine yield cigarettes and Russell[36] went so far as to suggest that tar intake is more likely to be reduced by developing low-tar, medium nicotine cigarettes.

1.9 Tissue Distribution

After absorption through the lungs, nasal mucosa or bronchial epithelium, nicotine is rapidly assimilated into the blood stream and distributed around the body. The presence of nicotine in various organs was first reported by Bonaventura Orfila who detected nicotine in the liver, kidney, lungs and blood of animals poisoned by nicotine. Schmiterlöw et al.[37,38] followed at short-time intervals the distribution of ^{14}C-labelled nicotine after a single dose of nicotine using wholebody autoradiography in combination with radioassay. These methods visualize the distribution of the radioactive isotope present, but as they are based only on the presence or absence of the radioactive indicator they give no hint whether it is the parent compound or its possible metabolites.

The picture of the distribution of radioactivity changes rapidly with time, hence it is important to note the exact time which has elapsed between the administration of nicotine and the tissues being autoradiographed. A mouse sacrificed 5 minutes after an i.v. injection of ^{14}C-nicotine showed a very high concentration of radioactivity in the brain, adrenal gland and kidneys. Some radioactivity was

also observed in the liver, heart muscle and salivary glands. However, with animals sacrificed some 30 minutes after i.v. injections, the radioactivity in the brain had decreased considerably. As the time between the i.v. administration of ^{14}C-nicotine and the sacrificing of the animal increased, the brain showed less and less radioactivity. This is the reason why Ganz, Kelsey and Geiling[39] found only traces of nicotine in the brain whereas Werle and Meyer[40] reported that the brain contained the highest amount of nicotine. Ganz et al.[39] made their determination 3 and 6 hours after i.v. injection of nicotine whereas Werle et al.[40] did theirs almost immediately after the nicotine was administered. In experiments with pregnant mice it was clear that nicotine freely passes the placental barrier.[37] Considerable amounts of radioactivity remain in the excretory organs of the mouse, i.e. the kidney, liver and gastric mucosa even 30 mins after nicotine administration.

Schmiterlöw and Hansson[37] also studied the superior cervical ganglion of the cat in greater detail. Many of the desired effects of nicotine occur in the brain due to nicotine action on the autonomic ganglia. They found that initially the radioactivity is localized in the ganglion cells and not the connective tissue. However, it was also apparent that some ganglion cells contained more radioactivity than others. Volle[41] found evidence that the ganglion cells are "functionally heterogenous" which is in agreement with the hypothesis of Shaw et al.[42] of functional differences among synapses.

1.10 Metabolism of Nicotine in Various Tissues

Mammalian metabolism of nicotine has been of interest for many years; not solely because of the popularity of cigarettes,where the metabolites of nicotine might be responsible for the effects produced by smoking, but as a pharmacological tool. Nicotine resembles important enzymatic constituents and natural amino acids, proline and hydroxy-proline, structurally.

Corcoran et al.[43] observed that only a small fraction of the absorbed nicotine is eliminated in the urine. Bennett et al.[44] from studies on dogs reported only 10% approximately of the administered ^{14}C-nicotine excreted unchanged. On the basis of animal experiments it can be concluded that this low excretion of nicotine probably arises from an active metabolism and does not indicate storage or excretion by other pathways.

Lautenbach[45] in 1876 was the first to show that passage of nicotine through the liver prevented its toxic effects in the dog. Animals with liver damage tolerate much lower levels of nicotine than healthy animals.[46] This also explains why ingestion has never been used as a method for the intake of tobacco.[20] Nicotine, after absorption through the gut, passes through the liver where it is metabolized, so preventing nicotine from getting to the brain in its active form. Absorption through the lung, mouth or nose has the advantage, as it bypasses the liver initially.

Miller and Larson[47] established in 1953 that tissues other than the liver were capable of detoxifying nicotine. Hansson and Schmiterlöw[48,49] have also investigated the biotransformation of nicotine in various tissues. They studied the contribution made

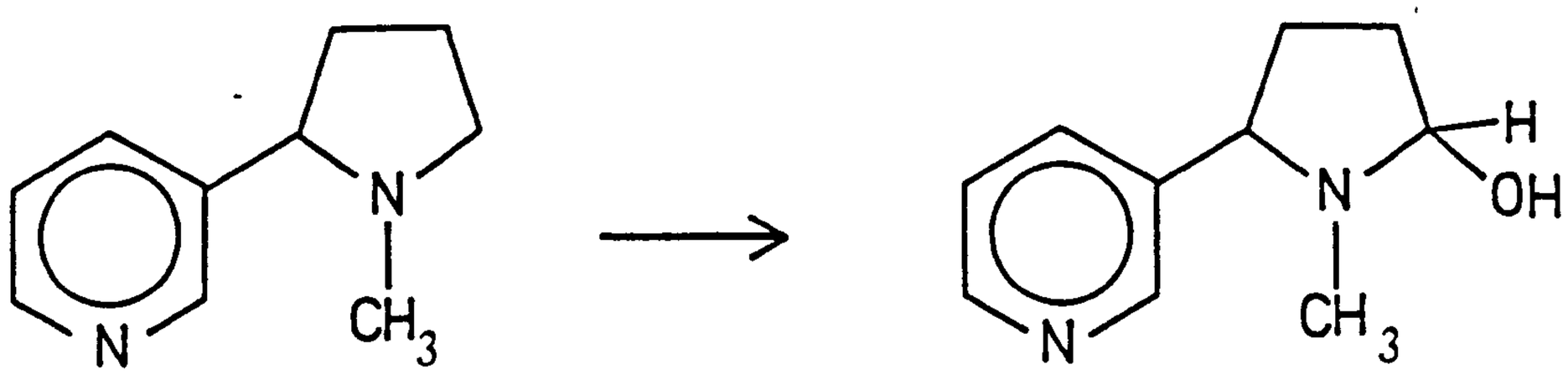
by different organs in the metabolism of nicotine. Tissues were removed from mice and rats at various times after the injection of ^{14}C-nicotine (in vivo studies) and the tissues extracted with chloroform. The radioactive compounds were then separated by paper chromatography. Various tissue slices were also incubated with radioactively labelled nicotine (in vitro studies). TLC was used to identify some of the breakdown products and measure their quantitative significances. These methods were not very selective and not sensitive enough to detect small amounts of radioactive compounds. From their experiments Hansson and Schmiterlöw showed that as time passes, more and more of the nicotine is transformed into metabolites, the main one being cotinine. The lungs and kidney, in addition to the liver, were found to metabolize nicotine, while the brain, diaphragm, spleen, stomach, small intestine and adrenal glands did not. The chloroform fraction from the liver extraction gave 5 radioactive compounds in addition to nicotine on TLC separation. The kidney produced two metabolites and $^{14}CO_2$; in the lung only one other metabolite was found, cotinine.

1.11 Proposed Mechanisms of Metabolism

Cotinine was the first distinctive mammalian metabolite of nicotine, identified in the form of its picrate and dipicrate by McKennis et al. in 1957.[50] This is the major in vivo pathway of metabolism in most species. The presence of cotinine has been confirmed in animal studies on the rabbit,[51,52] rat[53] and mouse[54] and in urine

from human subjects[55] after smoking or ingestion of nicotine. Conversion of nicotine to cotinine occurs in the lungs, kidney and liver, which has been established by in vitro studies involving tissue from several species.[48]

The mechanism for the formation of cotinine is complex. Hucker et al.[51] and McKennis et al.[56] have proposed different pathways for the formation of cotinine from nicotine. McKennis et al.[50,57] isolated γ-(3-pyridyl)-γ-methylaminobutyric acid from urine after the administration of nicotine and assigned it as an intermediate in the formation of cotinine, suggesting that it can be converted to cotinine by ring closure. This acid can spontaneously form its lactam, cotinine, at physiological pH and temperature. Later Hucker et al.[52] showed that little if any cotinine is formed in the liver microsomes by the mechanism put forward by McKennis et al. Hucker et al.[52], from in vitro experiments, proposed hydroxynicotine as a possible intermediate. Initial hydroxylation of nicotine in the alpha (α) position of the pyrrolidine ring would give the intermediate hydroxynicotine which in turn could be oxidized to cotinine.

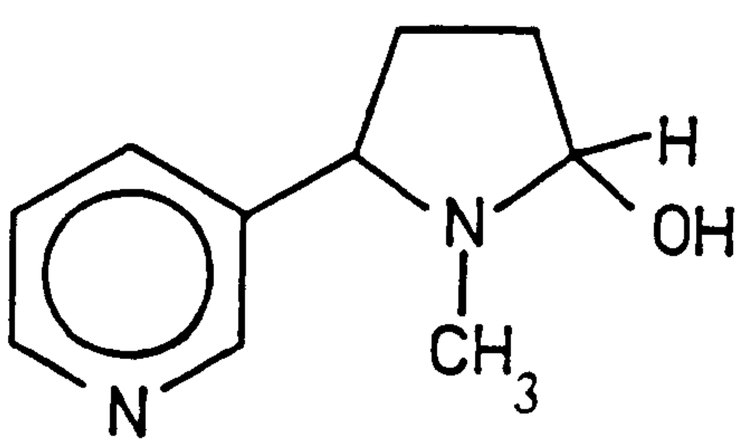


Nicotine

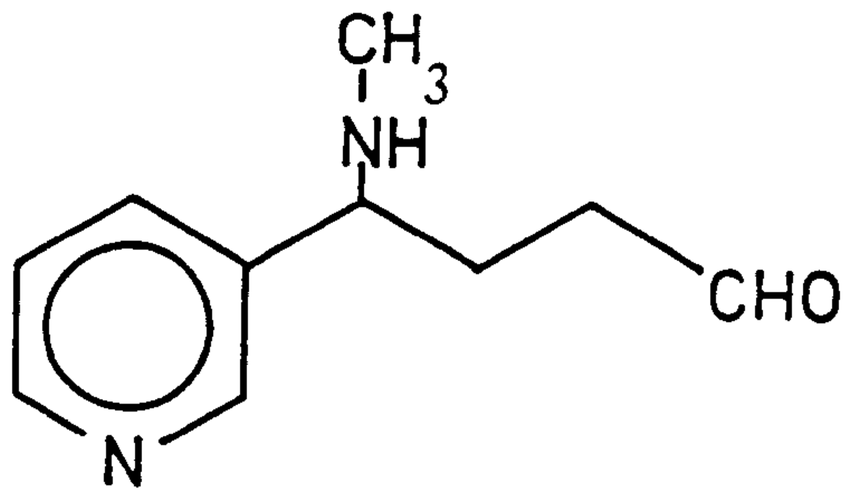
Hydroxynicotine

Cotinine

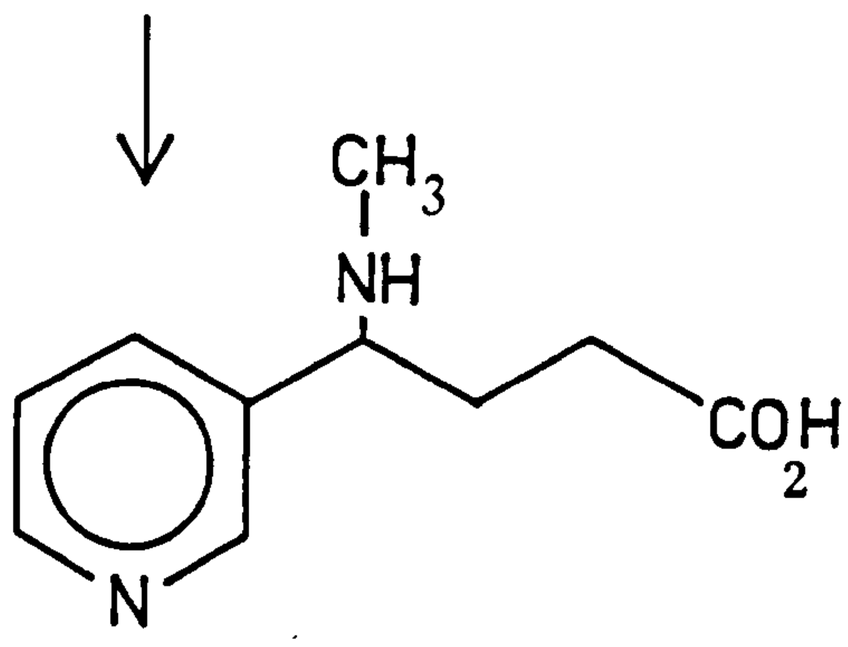
The acid identified by McKennis et al. can also be formed by the internal dealkylation of hydroxynicotine to form the aldehyde (as shown below) which can then be oxidized to the γ-(3-pyridyl)-γ-methyl-aminobutyric acid.[56]



Hydroxynicotine



γ-(3-pyridyl)-γ-methyl-amino butyraldehyde



γ-(3-pyridyl)-γ-methyl-amino butyric acid

Papadopoulos et al.[58] although providing additional evidence for the formation of cotinine from nicotine in rabbit tissues in vitro and in vivo were not able to detect either of the proposed intermediates.

Evidence of the formation of 8 major nicotine metabolites was presented by Papadopoulos et al.[58] which suggested more than one major pathway in operation for the metabolism of nicotine; an oxidative process, that yields cotinine as the major metabolite, and a demethylation

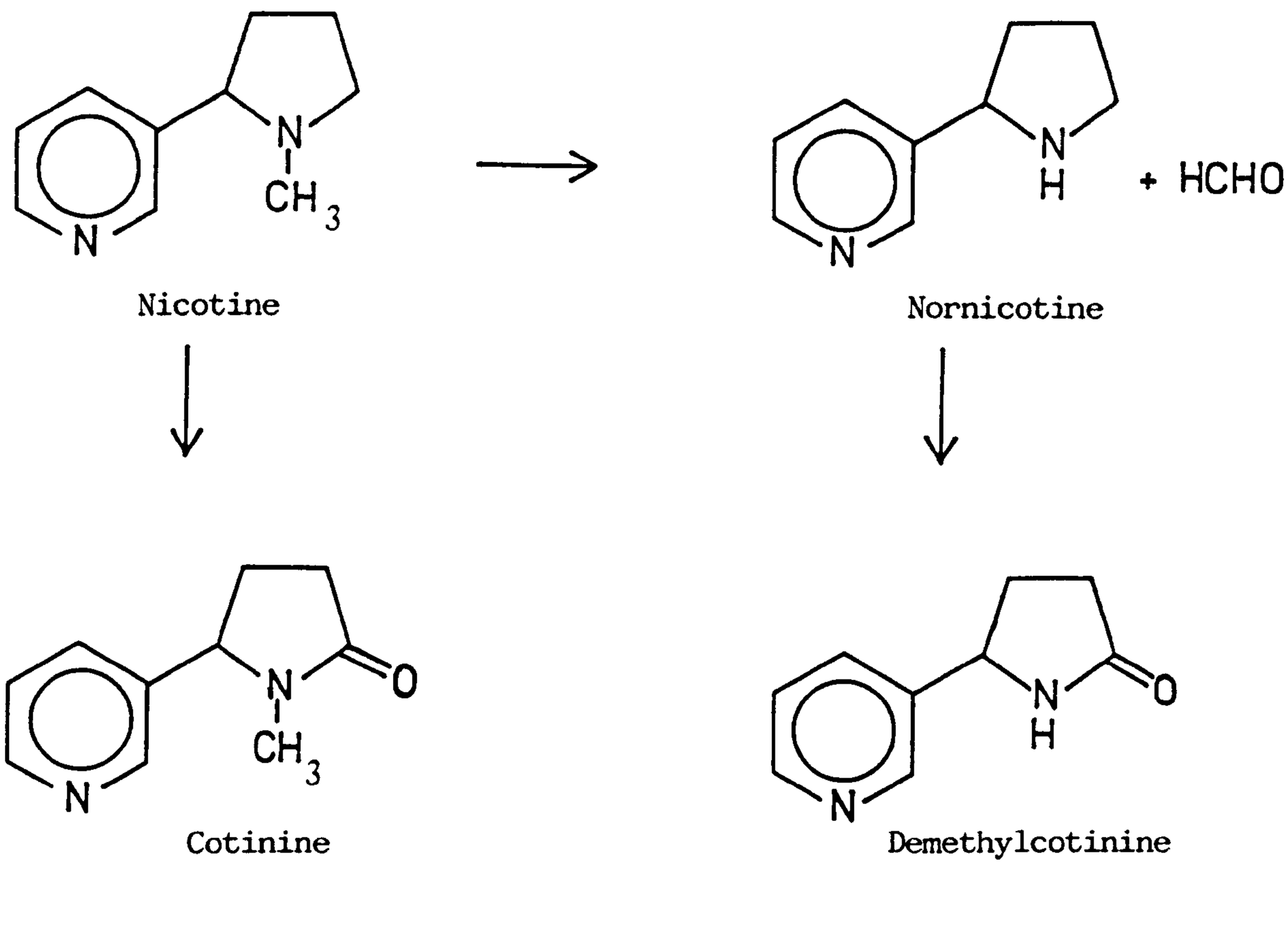
process, resulting in the formation of demethylation products of both nicotine and cotinine. The four metabolites positively identified by Papadopoulos[59] include cotinine, nornicotine, demethylcotinine and pyridylacetic acid. Nicotine 1'-N-oxide was tentatively named as one of the unidentified metabolites.

In Hansson and Schmiterlöw's[48] early experiments on the identification of nicotine metabolites using radioactively labelled nicotine, the presence or absence of demethylated products of nicotine metabolism could not be detected as the ^{14}C label was on the methyl group of the pyrrolidine ring.

Although Hucker et al. reported[52] in 1959 that the conversion of nicotine to nornicotine by a rabbit liver preparation was negligible, McKennis et al.[60] presented evidence three years later for demethylation in the metabolism of nicotine by the isolation of demethylcotinine in the urine of dogs following the administration of nicotine; and by the demonstration that the methyl group of nicotine is the precursor of expired CO_2 in the rat. From in vitro enzymatic studies, Papadopoulos[58] identified both metabolic nornicotine and demethylcotinine lending additional support to the findings of McKennis et al. for the demethylation pathways. In a later study, Papadopoulos[61] isolated and characterized nornicotine as a metabolite of nicotine. Demethylation of nicotine to nornicotine and the conversion of the latter to demethylcotinine was also demonstrated.

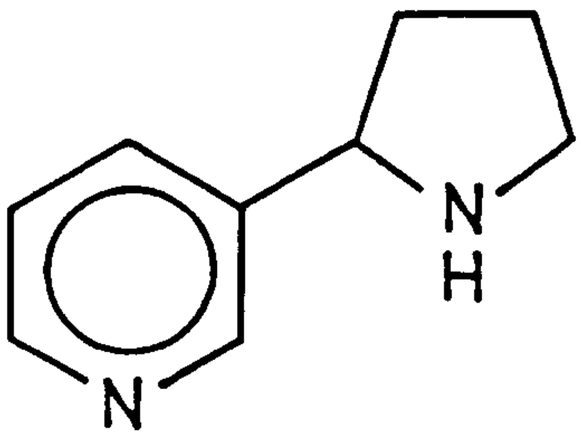
The mechanism of nicotine demethylation has not as yet been established. In vitro and in vivo studies have shown nornicotine formation from nicotine and demethylcotinine formation from nicotine and nornicotine. Demethylcotinine was detected after the administration

of cotinine to dogs, mice and rats. Even though it was not detected in humans after cotinine administration, it was present in the urine of smokers. Although McKennis et al.[62] favoured the simplest explanation for the formation of demethylcotinine, which would involve only the participation of cotinine or γ-(3-pyridyl)-γ-methyl-amino-butyric acid, Papadopoulos[61] considered there was lack of evidence for demethylcotinine formation from cotinine, and gave the following sequence of reactions in the metabolism of nicotine by the rabbit.

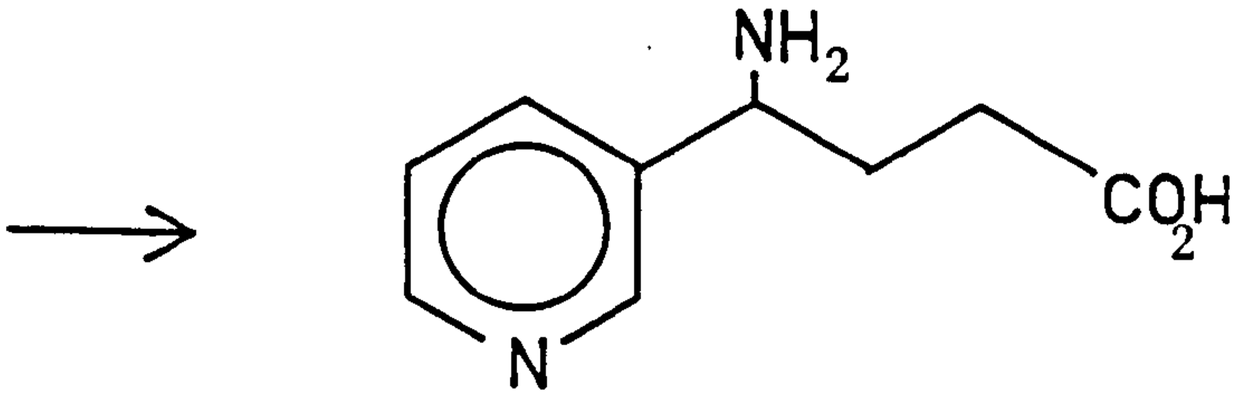


Werle et al.[63] also considered the possibility that nicotine was demethylated to give nornicotine. This opened up the possibility of additional metabolic pathways to demethylcotinine with nornicotine

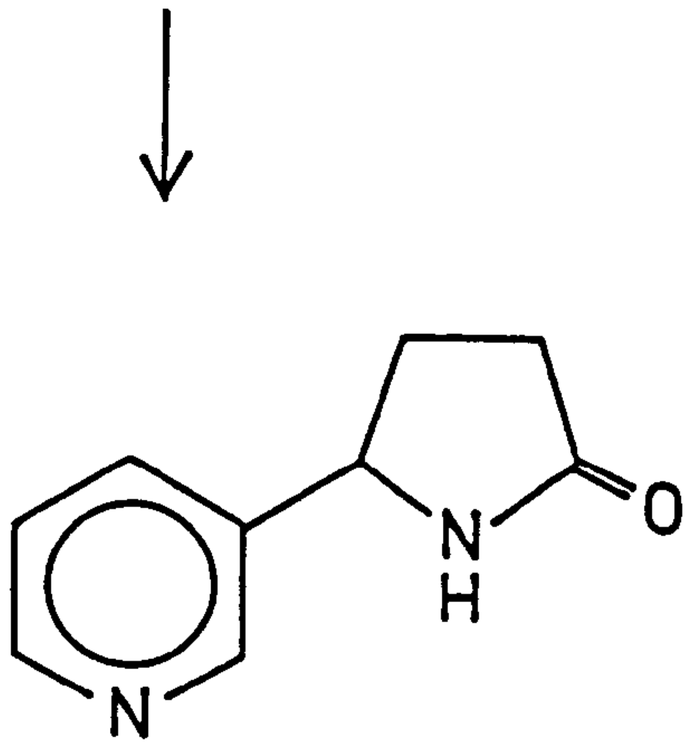
and possibly γ-(3-pyridyl)-γ-aminobutyric acid as intermediates and no involvement of cotinine.



Nornicotine



γ-(3-pyridyl)-γ-aminobutyric acid

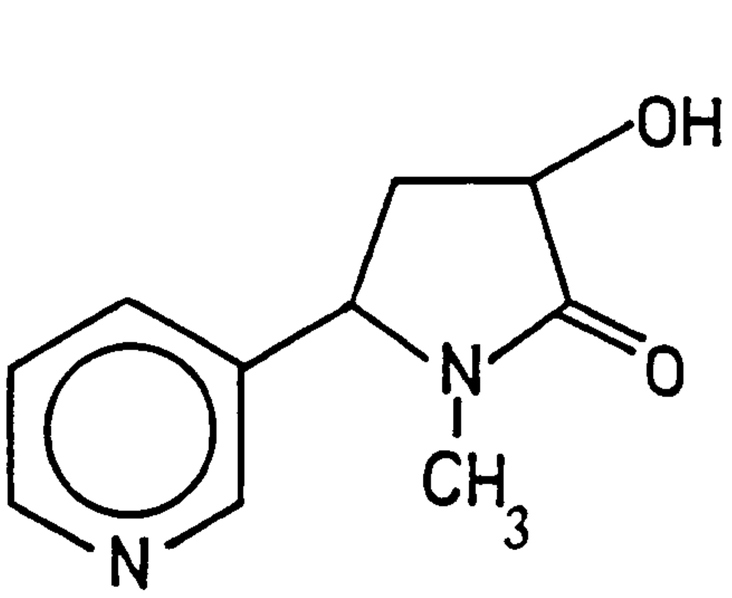


demethylcotinine

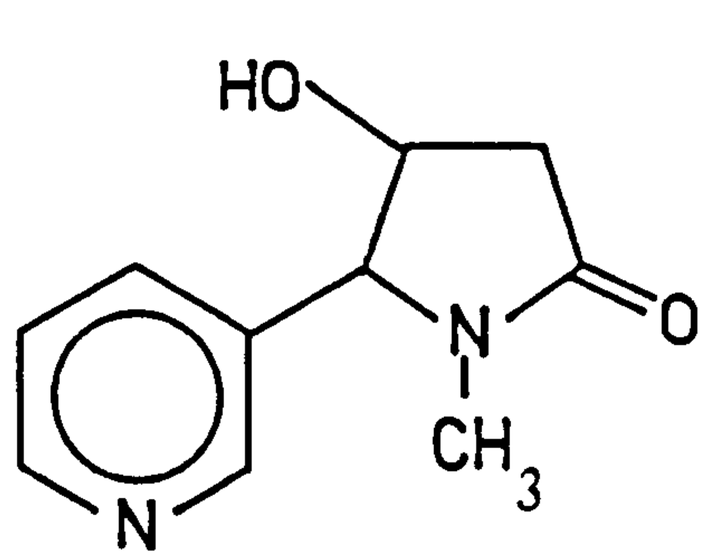
Nicotine-1'-N-oxide has been detected in the urine of smokers,[64] cats[65] and rabbits[61] and it is formed <u>in vitro</u> in hepatic and lung preparations from several species.[66] The formation of nicotine-1'-N-oxide is stereochemically governed. Booth and Boyland[64] reported that nicotine was enzymically oxidized into both isomers of nicotine-1'-N-oxide <u>in vitro</u>. The relative amounts of each isomer formed varied with different species and with different tissues. Reduction of nicotine-1'-N-oxide back to nicotine <u>in vivo</u> and <u>in vitro</u> has also been reported.[67] It is thought that reduction is due to metabolism by the gut flora or by enzymes of the intestinal wall. Oral

administation of nicotine-1'-N-oxide resulted in urinary excretion of both nicotine and cotinine indicating that reduction did occur. On intravenous injection of nicotine-1'-N-oxide, it was completely recovered in urine, unchanged. An important route for the conversion of nitrogenous foreign compounds into water soluble metabolites may be by the N-oxidation of ring N-atoms.[68] N-oxidation of cotinine has also been reported, in this case it is the pyridine nitrogen which is oxidized. Cotinine-N-oxide has been detected <u>in vivo</u> in the monkey.[69]

Hydroxycotinine was found in smokers' urine[55] and has also been detected in urine from dogs,[70] rats,[71] mice,[72] monkeys[73] and humans after cotinine administration.[74] After synthesizing the most likely isomeric forms of hydroxycotinine,



and



their optical rotation and melting points were compared with metabolic hydroxycotinine and the structure was tentatively described as having the C-hydroxyl group in the α position to the carbonyl group in the pyrrolidone ring although it was not designated cis or trans.[75]

Other metabolites of nicotine which have been identified are thought to arise by cleavage of the pyrrolidone ring of cotinine

or demethylcotinine. Hydrolysis of the amide linkage in cotinine results in the formation of γ-(3-pyridyl)-γ-methylaminobutyric acid and oxidative 1,2 cleavage gives γ-(3-pyridyl)-γ-oxo-N-methylbutyramide.[23]

The pyrrolidone ring can undergo a number of other changes which lead to the formation of 3-pyridylacetic acid in the dog,[70] the mouse[72] and man.[76] No evidence exists for further oxidation of 3-pyridyl acetic acid to nicotinic acid.[23]

Many different routes are involved in the detoxification of nicotine in the body. It is far more diverse and complex than was initially thought. At least eighteen metabolites[23] have already been identified but still less than 30% of the total urinary metabolites can be accounted for (see figure 1.3).

1.12 Excretion

Ganz, Kelsey and Geiling[39] from studies in rats and mice found the main excretory route to be the urine. This has been confirmed by many other studies including that of Bennett et al.[44], McKennis et al.[77], using dogs, and Turner[78] who reported that urinary excretion in the cat accounted for 90% of the total (multiple) dose in 72 hours. Ganz et al.[39], using randomly labelled ^{14}C-nicotine, stated that approximately 50% of the radioactivity of a single dose was excreted within 6 hours after injection. The urinary excretion of radioactivity in the rat was evident almost immediately after injection and almost all the radioactivity was excreted during the course of 16 hours. Each of the studies however report a different percentage for the

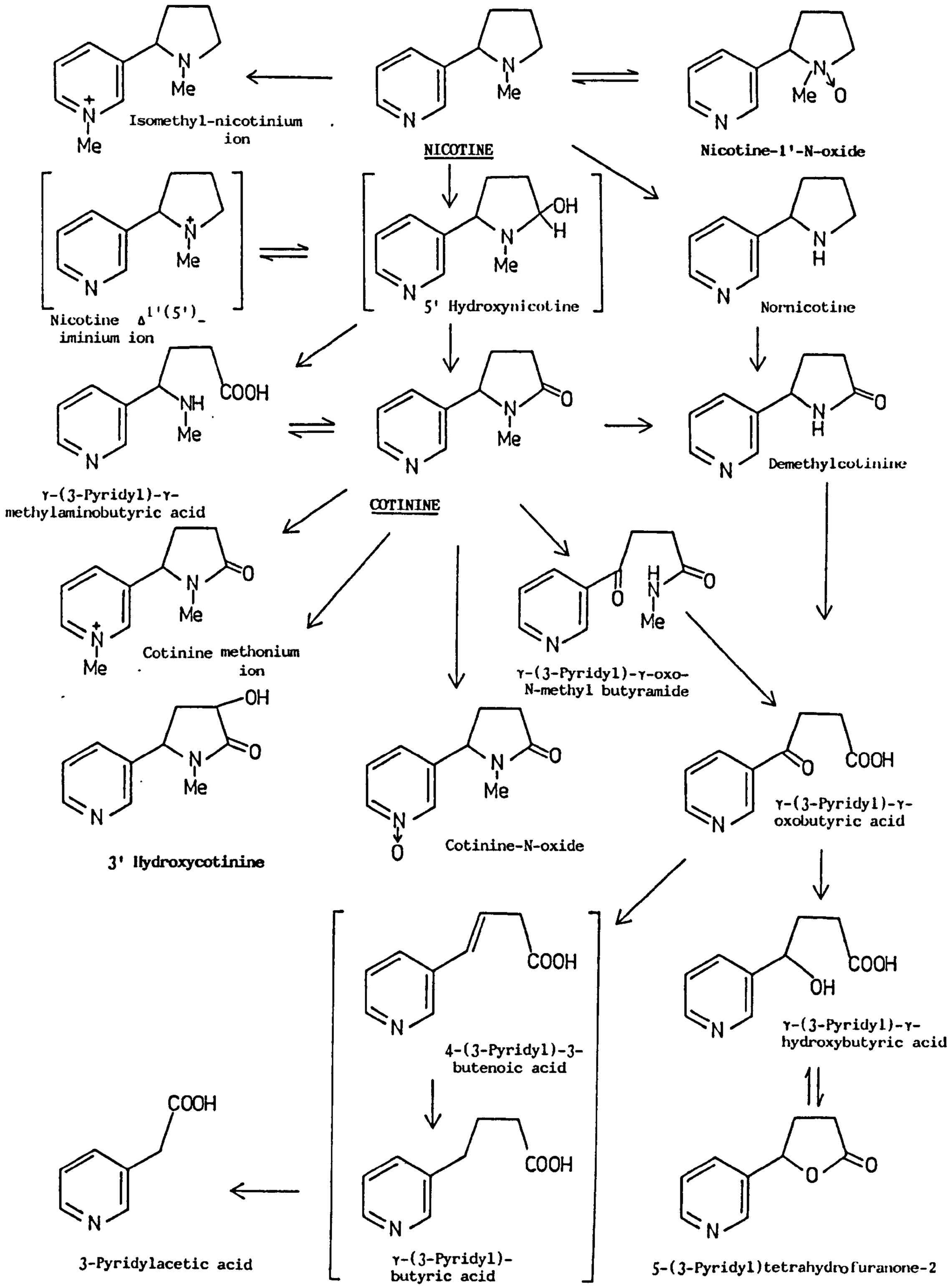


Figure 1.3: Metabolic Routes for the Metabolism of Nicotine

excretion of unchanged nicotine. McKennis[50] found approximately 10% of the radioactivity present as nicotine whereas Turner[78] reported only 2.5% and Ganz et al.[39] reported a value of 25%, although it should be noted that different species were studied in each case.

As with absorption, the excretion of nicotine is pH dependent. One pKa value for nicotine is 7.9. Under acidic conditions a very small percentage of nicotine is present as the undissociated (free) base, whereas at more alkaline pH values, a considerable proportion of the free base is present. The undissociated base nicotine is lipid soluble and it can permeate cell membranes readily. It can be absorbed not only through the lungs but also very quickly through the bladder and renal tubules.[79] Therefore, at an acidic pH below 6.0 ($\sim$ 5.5),[79] the nicotine, in ionized form, cannot be reabsorbed through the renal tubules. The excretion of unchanged nicotine is therefore enhanced to perhaps as much as 30-40% of an administered dose. At alkaline pH values of 7 or greater ($\sim$ 8)[79], the nicotine now present mostly as free base can be reabsorbed through the renal tubules and also the bladder. This has been verified when the urine has been acidified for example by the intake of vitamin C, nicotine excretion is enhanced. Intake of sodium bicarbonate switches the urine pH to the alkaline side causing a decrease in the urinary excretion of nicotine. It seems plausible that as a result of the increased reabsorption of nicotine there may be a decrease in the number of cigarettes needed to maintain the plasma nicotine level. From plasma nicotine profiles in subjects with alkaline urinary pH, a "rebound" in the curve was noted after smoking stopped - due to reabsorption. Russell and Feyerabend[20] found that plasma nicotine

levels were affected by changes in the urinary pH. In fact, the rate of renal clearance of nicotine can influence the intake of nicotine through smoking. Benowitz et al.[80] found that the smoker partially compensated for the increase in the rate of elimination of nicotine under acidic urine conditions, by increased nicotine intake. Most smokers smoked the same number of cigarettes but may have achieved increased intake by changing their puff profile or depth of inhalation. However, only half the excess urinary nicotine loss was replaced. Unfortunately, Benowitz et al. found that under alkali treatment there was only a small decrease in nicotine excretion over a 24 hour period compared with the controls, therefore variation in cigarette smoking habits are unlikely. Fix et al.[81] had proposed urinary alkalization. to decrease renal clearance of nicotine which in turn would cause a reduction in nicotine consumption.

Beckett et al.[82] concluded that urinary excretion of nicotine was unaffected by urine flow but Matsukura et al.[83] thought nicotine excretion was volume dependent, and Feyerabend and Russell[84] found it to be proportional to the rate of urine flow, under acidic conditions.

The influence of both pH and urine flow rate on the excretion of cotinine is also a source of disagreement in the literature. Beckett et al.[82] reported cotinine excretion to be slightly pH dependent and also volume dependent. This view was supported by Matsukura et al.[83] who found urinary excretion of cotinine was affected more noticeably than nicotine by changes in urine flow. However this finding was contradicted in another paper by the same author[85] which reports 'no correlation between urinary cotinine excretion and pH

or urine flow after smoking'.

Nicotine-1'-N-oxide, being a highly polar water soluble compound, not easily absorbed in the body at any pH, was found to be independent of changes in both urinary pH and flow rate.[82]

From all the observations, pH is undoubtedly the more influential factor affecting the excretion rates. Nicotine excretion is very rapid, the peak concentration occurring about 15 mins[82] after smoking a single cigarette. There is a tendency for the plasma nicotine in habitual smokers to increase over the first few hours during which smoking takes place, after which it tends to remain fairly constant. This "steady state" could be the result of self-titration by the smoker. There is no significant accumulation of nicotine in the body from one day to the next ($t_{\frac{1}{2}}$ nicotine < 30 mins).[86]

The peak of cotinine excretion could be up to 4 hours[66] after smoking a single cigarette. Although cotinine (pKa 4.5)[87] is essentially not ionized in blood at pH 7.4, the free base is not soluble in lipids so its rate of distribution into body tissues is slow. This could explain why cotinine remains in the blood system for much longer than nicotine ($t_{\frac{1}{2}}$ cotinine 30 hours).[80] The rate of urinary excretion of cotinine was much slower than either nicotine or nicotine-1'-N-oxide. Nicotine and nicotine-1'-N-oxide have similar excretion profiles under maintained acidic urinary pH. The fraction of nicotine-1'-N-oxide excreted by smokers in 24 hours under normal conditions was approximately half that of cotinine excreted.[82]

Non-smokers may passively inhale other people's cigarette smoke (passive smokers). Horning et al.[88] reported that when non-smokers and smokers shared a laboratory area, nicotine was found to be present

in non-smokers' urine to the extent of about 5% of that observed for smokers. It is probable that the route of nicotine transfer for non-smokers is through room air and in fact nicotine was found in the lab air by Horning et al. This finding is in accordance with other studies such as that by Feyerabend et al.[89] On analysis of plasma samples, a peak corresponding to that of nicotine was found in non-smokers' samples; they identified this peak as nicotine. This was later confirmed by Falkman et al.[90] using a mass spectrometric method in which all the fragments characteristic of nicotine were present. Although the same peak was observed earlier by Isaac and Rand,[86] it was considered to be an interferant and the levels found in non-smokers, the so-called blanks, were subtracted from the values of blood nicotine concentrations obtained for smokers. Unfortunately the blood nicotine concentrations found in non-smokers are not constant. According to Russell et al.,[91] the difference between those non-smokers who have been heavily exposed to tobacco smoke and those who have not is highly significant statistically ($P < 0.001$). In a more recent paper, Feyerabend et al.[92] showed that the levels of nicotine excreted by non-smokers who had been exposed to tobacco smoke in the period prior to sampling were similar to those levels found in light smokers, who smoked up to three cigarettes before sampling.

1.13 How Smoking affects the Metabolism of Nicotine and hence its Excretion

Nicotine is extremely toxic, only small quantities of it can be tolerated by man and so it is broken down and eliminated rapidly from blood after smoking. Beckett et al.[93] have suggested that smoking induces nicotine metabolism. Smokers certainly develop a tolerance for nicotine — at first their bodies tend to reject it and the unpleasant effects which it can produce, such as dizziness and nausea, however after the first few cigarettes these effects disappear. In a study on the effects of smoking on nicotine disposition Kyerematen et al.[94] found that both plasma nicotine and cotinine concentrations decay faster in cigarette smokers than in non-smokers. This is in contrast to habituated snuff dippers who were found to have similar mean plasma nicotine $t_{\frac{1}{2}}$ values and habituated pipe smokers where $t_{\frac{1}{2}}$ values were only very slightly shorter than in 'naive' users.

From in vitro studies on the effect of nicotine treatment on the metabolism of nicotine in the mouse liver, Stalhandske et al.[95] found an increase, though not a significant increase, in the formation of cotinine. They reported a dual effect of nicotine treatment on liver metabolism. Nicotine may both stimulate and inhibit its metabolism depending on the dose administered and the duration of treatment.

The chemical(s) in tobacco smoke responsible for the enhancement of nicotine and cotinine metabolism are not known although polycyclic hydrocarbons have been suspected. Pantuck et al.[96] investigated the effect of cigarette smoking on phenacetin metabolism in rats and humans. They also singled out one of the polycyclic hydrocarbons

present in cigarette smoke and examined whether it, in particular, had any effect on the phenacetin metabolism in the rat. They found that the mean concentrations of phenacetin in the plasmas of smokers were considerably lower (∿ 20%) than in non-smokers, leading to the conclusion that cigarette smoking stimulates the metabolism of phenacetin in man. In rats, treatment with 3,4-benzpyrene injected intraperitoneally in corn oil also enhanced the metabolism of phenacetin. Pantuck et al.[96] also noted that the plasma concentration of the major metabolite of phenacetin was similar in both smokers and non-smokers. This may indicate enhanced metabolism of the metabolite or promotion of alternative routes of metabolism.

Results which may appear to contradict the evidence for increased metabolism of nicotine in smokers were obtained by Haines et al.[97] They found that the mean plasma nicotine concentrations were higher for habituated than for 'naive' smokers. These results can be explained by the fact that habituated smokers are capable of extracting more nicotine from inhaled smoke than 'naive' smokers, so giving higher concentrations of nicotine in the bloodstream.

As cigarette smoking can affect the metabolism of some drugs (e.g. phenacetin), it is also possible that other drugs can cause changes in nicotine metabolism. Adir et al.[98] have investigated the effect of ethanol pretreatment on the metabolism of nicotine in rats. This is a very interesting and important combination of drugs as a statistically significant correlation has already been established between high alcohol (ethanol) consumption and heavy smoking.[99,100] The results showed plasma nicotine levels in rats pretreated with ethanol were significantly lower than in the control

group. The plasma cotinine concentrations were higher during its formation and reached a maximum in a shorter time than in the control group which was pretreated with sucrose solution. The maximum plasma cotinine concentration was similar in both groups but the concentrations during elimination were always lower in the ethanol pretreated group. The lower plasma nicotine levels which resulted from ethanol pretreatment may cause the smoker to increase the number of cigarettes smoked which would lead to a positive correlation between alcohol consumption and number of cigarettes smoked.

1.14 Other Factors which affect the Metabolism of Nicotine

In addition to the effects which influence the metabolism of nicotine, already discussed, Beckett et al.[101] reported evidence supporting the existence of sex differences in the metabolism of nicotine in humans. Nicotine was administered to groups of male and female smokers and non-smokers by i.v. injection, after which Beckett et al. found that male smokers fell into two distinct groups, one of which showed a lower percentage of both nicotine and cotinine excreted as compared with non-smoking males, and another group which showed a higher percentage of cotinine excreted but similar amounts of nicotine to those in male non-smokers. Female smokers excreted more cotinine but less nicotine than female non-smokers. The percentage of each excreted was higher than for male smokers. The male non-smokers excrete less nicotine but more cotinine than female non-smokers showing that the metabolism of nicotine is sex dependent. The metabolism of nicotine in smokers is yet more complicated. The excretion profile for female smokers showed increased nicotine

metabolism favouring the formation of cotinine or where further metabolism of cotinine is prevented. In male smokers, where the excretion of both nicotine and cotinine is low, it is possible that cigarette smoking has enhanced the metabolism, whereas when the excretion of cotinine is high, this could be either the result again of further metabolism of cotinine or the formation of cotinine being emphasised as the metabolic pathway.

Age may also affect metabolism, although this has not been studied in depth. Stalhandske et al.[102] found that cotinine formation improved with age in a study with mice.

1.15 Correlation between the Numbers of Cigarettes smoked and the Blood/Urinary Nicotine Values

A relationship between the number of cigarettes smoked or the nicotine yield of cigarettes and blood or urinary nicotine concentrations has been sought. Russell et al.[103] have reported a correlation between blood nicotine concentration and the nicotine yield of cigarettes. Although the correlation was significant, it was low, accounting for only 4.4% of the variation in blood nicotine concentrations. In a later paper, Feyerabend et al.[92] suggested that the plasma nicotine level just after smoking a cigarette may depend more on the smoking profile, the way the cigarette is smoked, than on its nicotine yield or the number which have been smoked over the previous few hours. Nicotine yields of cigarettes are measured by smoking machines but people do not smoke like the smoking machine.

The lack of correlation between plasma or urinary nicotine concentrations and number or nicotine yield of the cigarettes is less surprising when all the factors such as absorption, metabolism and excretion are considered. pH Effects, sex differences in metabolism and the fact that smoking itself can enhance breakdown have already been reported.[66] Individuals' observed nicotine levels can also show variations due to differences in lung efficiency, age, and other, perhaps genetic, factors. Some workers[27,104-107] have switched to using blood cotinine concentrations as a measure of nicotine exposure. The long half-life of cotinine means that its concentration in blood varies very little over the course of a smoking day. It is not a perfect marker as there will be individual variations in the conversion of nicotine to cotinine. Quantitative measurement of nicotine and more of its metabolites might give a clearer picture of the relationship between the number of cigarettes or nicotine yield, and the resulting concentration levels present in blood or urine after smoking.

A striking example of the possible use or importance of the quantitative determination of nicotine metabolites was cited by Gorrod et al.[108] Analysis of two groups of subjects, one with confirmed urinary cancer and the other a control group of normal subjects, showed that there was a significantly higher ratio of cotinine to nicotine-1'-N-oxide in cancer patients than in the control group. It was not known whether the change in the ratio was a possible cause of the disease or as a result of it or its treatment.

1.16 Effects of Nicotine: How it works

There is little doubt that people smoke for the nicotine and the effects that smoking doses of nicotine produce on the central and peripheral nervous system. Continuous cigarette smoking is considered an attempt by the smoker to maintain the plasma nicotine level.

Nicotine can have an effect on almost every organ in the body either directly or indirectly. Nicotine is a ganglionic-blocking agent, that is it can block nerve impulse transmission across synapses in the autonomic ganglia. Nerve impulses are transmitted by the release of acetylcholine at the synapse. Ganglionic-blocking drugs do not interfere with the liberation of acetylcholine or with its rate of synthesis or hydrolysis, they mimic the action of acetylcholine. There are two main classes of ganglionic-blocking agents. Nicotine has been classed as one of the depolarizing drugs. This type of drug initially causes stimulation of the postsynaptic receptors, the stimulation persists and so results in depolarization and depression of the synaptic transmission. Nicotine also causes the release of amine transmitters such as noradrenaline, serotonin and possibly dopamine which affect the whole nervous system.[24]

In general small doses of nicotine stimulate the central nervous system (CNS), whereas with large doses depression follows stimulation. Doses or concentrations of nicotine capable of producing depression or paralysis are never involved in normal smoking by habitual smokers.

Although of interest, the pharmacological effects of nicotine should not be confused with those of tobacco smoke. Nicotine is a very important factor in tobacco usage but it is clearly not the

only one involved.

In many pharmacological studies, the dosage or concentration of nicotine used is very much greater than that which can be extracted even by the habitual smoker. Also the route of administration may differ, intravenous injection rather than inhalation may be used due to the more precise control it gives the experimenter over the dose administered. However these changes may not give compatible results.

1.17 Health Consequences of Smoking

Many investigations have examined links between particular factors and diseases and, although links are often found, drawing conclusions about causality can still be very difficult. It has been reported that lung cancer affects smokers more often than non-smokers and heavy smokers are most at risk. Other factors such as air pollution or occupation may also contribute to the end result but smoking is considered to be a primary cause.[1] Investigations have also established strong associations between smoking and other cancers such as those of the oral cavity, larynx, oesophagus, pancreas, bladder and kidney.[109] Coronary heart disease is a more frequent cause of death in smokers, particularly cigarette smokers, than in non-smokers. However many other factors such as diet and stress are also associated with heart disease and it quite often affects non-smokers.[1] In 1957 Simpson[110] showed that women who smoke during pregnancy tend to give birth to underweight babies. Butler et al.[111]

confirmed the association between cigarette smoking during pregnancy and reduced birth weight and added that it also increased late foetal and neonatal mortality rates.

The form of tobacco use is an important factor in the risk of developing diseases associated with smoking. Cigarette smokers are approximately ten times more likely to get lung cancer than non-smokers. In contrast, as most pipe and cigar smokers do not inhale, their risk of developing lung cancer is decreased when compared to cigarette smokers but it is still higher than for non-smokers.[109] Active smoking with inhalation is a very effective method of getting smoke into the lungs. It consists of drawing in a small volume of smoke (an average of about 35 cm^3 in the case of cigarettes) into the mouth, whereafter it is drawn into the lungs. Whether considering gases or particles, this technique draws the inhaled substance further into the lungs than if it were distributed throughout the whole volume of tidal air. A puff of cigarette smoke is completely washed into the depths of the lungs by the following tidal air.[12] Chewing tobacco and snuff dipping[112] result in an increased risk of oral cancer and cancer of the cheek and gum.

Several prospective studies on the association between smoking and disease and smoking and mortality are listed in table 1.3. The report by Hammond[116] gave support to the conclusion that for all categories of current smokers, risk of death from cancers of the lung, larynx, oral cavity, bladder and kidney was significantly higher. Coronary heart disease was also found to be higher in cigarette smokers than in non-smokers.[113,116] Death rates of current smokers increased with the number of cigarettes smoked per day, the degree of inhalation

TABLE 1.3: Prospective Studies

Ref.	Authors	Participants	Year started	Male participants	Female participants
113	Doll & Hill	British physicians	1951	40,000	6,000
114	Hammond & Horn	White males	1952	187,783	-
115	Dorn	US veterans	1954	290,000	< 1%
116	Hammond	Men and women	1959/60	1 million	562,671

and starting the habit at a young age. Death rates of ex-cigarette smokers decreased with the length of time since they last smoked. However, some unexpected figures were uncovered. Lung cancer death rates in Japan, USA and among white male South Africans were much lower than predicted from their cigarette consumption. Clearly other factors besides smoking are involved in the initiation of lung cancer.

1.18 Passive Smoking and the Health Consequences

Over recent years, concern has grown over the exposure of the non-smoker to sidestream cigarette smoke and the health hazards associated with inhalation.[117] Nicotine which has been used as a tracer for the particulate matter has been identified in the urine of non-smokers although the levels excreted were lower than those found in smokers. Carbon monoxide levels in blood measured as carboxyhaemoglobin (COHb) have also been cited[118] as an indication

of exposure to tobacco smoke. Elevated levels of COHb have been found in non-smokers exposed to a closed atmosphere heavily contaminated with tobacco smoke. However smoking is not the only factor which can cause raised COHb levels; industrial and domestic pollution and leaking motor car exhausts can also contribute.[119]

Sidestream smoke is much less complex chemically, the minor constituents of mainstream smoke being much less prevalent. There are some compounds which are present in higher amounts including ammonia, nitrogen oxides and total particulate matter (including nicotine). The passive smoker inhales the sidestream smoke after it has been diluted with air and lost some of the particulate matter through retention on walls, floors and other surfaces. Unlike the smoker, who inhales a concentrated 35 cm^3 of smoke, the passive smoker inhales the environmental tobacco smoke distributed throughout the tidal air and if nasal breathing is used, many more particles will be filtered out in the nose.[12] Bridge and Corn[120] found that the levels of CO from tobacco sidestream smoke are not hazardous to non-smokers but the concentrations of particulate matter emitted can exceed the recommended maximum concentration for suspended particulate matter. It is important to note that many studies involve exposure to environmental smoke far in excess of any real life situation.

Many people find smoke irritating, but for people already suffering from a heart condition or asthma, exposure may have more serious consequences.[121] Passive smokers with angina need less exercise time to bring on pain, even in a well ventilated room.[122]

Some very interesting epidemiological studies have been carried out on the effects of passive smoking on one member of a spouse pair.

Correa et al.[123] found an increased risk of lung cancer among non-smoking husbands or wives when the other partner was a heavy smoker. The effect of the smoking habits of one partner on the lung cancer risk of the other was first reported by Hirayama.[124] The study was carried out in Japan, a traditional society where 75% of men but only 15% of women smoke. There was a statistically significant correlation between the amount the husband smoked and the mortality of the non-smoking wife from lung cancer.

A similar study in Greece[125] again reported a statistically significant association beween the husband's smoking habits and the wife's lung cancer risk. However Garfinkel[126] on comparing non-smoking women married to non-smoking husbands with non-smokers married to smoking husands in the USA found very little, if any, increased risk of lung cancer.

Exposure of women in Japan and Greece to other people's smoke is probably very different from that in the USA. In Greece smoking was uncommon among women until about 1960 whereas many men were already habitual smokers. Therefore trends in the small effects of passive smoking may be much more easily distinguished. Unfortunately, due to the small numbers of smoking wives it was impossible for a comparison between the lung cancer rates for the active smokers and those for the passive smokers to be made.

In another study by White et al.,[127] testing the small airways function, it was found that not only did non-smokers exposed to a smoky environment score less, but also that they fell into the same risk category as smokers who do not inhale and light smokers who inhale between 1 and 10 cigarettes per day.

1.19 Chemicals Associated with the Health Risks

Many of the thousands of chemicals in smoke are a potential health hazard, some are known to be carcinogenic e.g. hydrogen cyanide, acrolein[1] and nitrosoamines.[128,129] However it is not known if any of these compounds, most present only in infinitesimal trace amounts, are the cause of or even contribute to the cause of diseases associated with cigarette smoking. Although they may not be present in sufficient quantities to initiate cancer in smokers, they may reinforce one another's cancer-causing potential or produce irritation, so making the tissue more prone to cancer.[1]

Nicotine and carbon monoxide[130] have come in for particular scrutiny. Nicotine is known to have cardiovascular effects. Changes in blood pressure and pulse rate have been recorded.[131] Nicotine causes the release of catecholamines which alter cardiac activity. It produces an increase in the amount of free fatty acids and it may lead to increased platelet adhesiveness.

Carbon monoxide has a very high affinity for haemoglobin. The formation of carboxyhaemoglobin results in a reduction in the availability of oxygen.

Free radicals are an extremely reactive species and it is known that they can cause cell damage. The 'tar' fraction contains free radicals but Pryor et al.[132,133] have now identified their presence in the gas-phase. Normally the free radicals are short-lived; they are so reactive. However, Pryor et al.[133] found that they were still present in smoke even a few minutes after the smoke had formed. This has led to the conclusion that they were forming in the smoke and can therefore form in the lungs. Nitric oxide and various alkenes,

both present in cigarettes and air, have been suggested as a possible combination of chemicals suitable for the production of free radicals in cigarette smoke.

The possibility that the metabolites of nicotine could be carcinogenic was of particular interest after the discovery of nicotine-1'-N-oxide. N-oxide derivatives were suspected in the origin of cancers after the discovery that the transformation of purines into their N-oxides gave rise to potent oncogenic agents.[134] Indeed, Booth and Boyland[135] reported that cotinine induced intestinal tumours in rats.

1.20 'Safer Smoking'?

It would obviously be much easier to make smoking safer if the harmful constituents were known and could then be removed or their concentration reduced to a minimum.[136] The tar content has been singled out as being the most lethal, but, as we have already mentioned, the low tar, low nicotine yield filter cigarettes now being produced do not necessarily benefit the smoker, who can compensate for reduced nicotine/tar levels by 'self titration'.[29]

The Framingham Heart Study[137] reported that smoking low tar, low nicotine yield filter cigarettes did not result in a lower risk of myocardial infarction than smoking cigarettes containing larger amounts of these substances. The explanation put forward was that filter cigarettes produce more CO than plain ones, although this was not supported by the findings of Kaufman et al.[138] There has

been a reduction in the incidence of lung cancer in smokers but Russell et al.[103] suggest that it may have more to do with the reduced carcinogenicity of tobacco tar over a number of years than switching to filter cigarettes with low tar and low nicotine yields. Robinson et al.[32] have reported that when smoking low tar/nicotine yield cigarettes, the degree of compensation was not only almost complete for nicotine but also for CO and HCN. To counteract this self-regulation,[139] Russell et al.[34,36] have suggested low tar, low CO cigarettes with a medium rather than a low nicotine yield. There is no evidence that other components of tobacco smoke such as tar and CO are intrinsically rewarding. Gust et al.[17] lend support to this hypothesis as it was found that lung exposure to cigarette smoke decreased when high nicotine cigarettes were smoked. However, this may not be the answer. Nicotine affects the pulmonary function[140] and the cardiovascular system and may adversely affect the foetus.

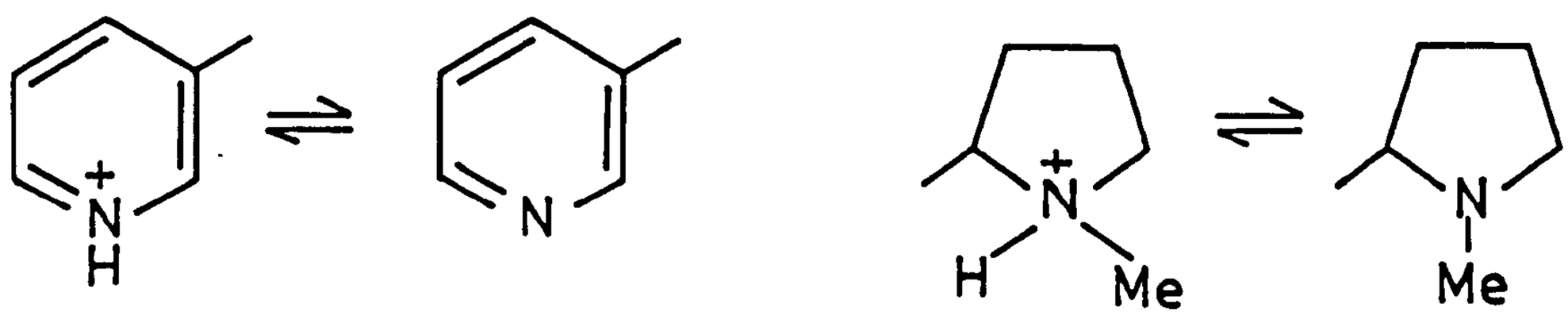
Another possible solution may be the deliberate use of irritants e.g. acrolein to help reduce the inhalation of smoke.[34] That compensation is often incomplete is thought to be due to the smoker finding the necessary increased smoke exposure too irritating.

The consequences of these studies and hypotheses are unknown and have not yet been tested.

1.21 Chemistry of Nicotine and its Metabolites[20,141]

Nicotine, (S)-3-(1'-methyl-2'-pyrrolidinyl)-pyridine, has the empirical formula $C_{10}H_{14}N_2$ and is a water-clear liquid, one of the few liquid alkaloids, boiling under atmospheric pressure at 246°C. Upon exposure to air it turns brown emitting the characteristic aroma of tobacco. It is miscible with water, alcohol and ether. Natural nicotine is lævorotatory, the nicotiana species always yielding the optically pure l-nicotine ($[\alpha]_D^{20} = -169°$).

Nicotine has two dissociation constants, one for the pyrrolidine and the other for the pyridine nitrogen, see figure 1.4. In blood at pH 7.4, 37°C, the pyridine nitrogen of nicotine exists primarily as the uncharged form, while the pyrrolidine nitrogen has both ionized and unionized species.[142] Approximately 69% exists as the charged form at this pH, so the predominant form of nicotine is the ionized pyrrolidine structure.



$pK_a \sim 3.12$ $pK_a \sim 7.8$

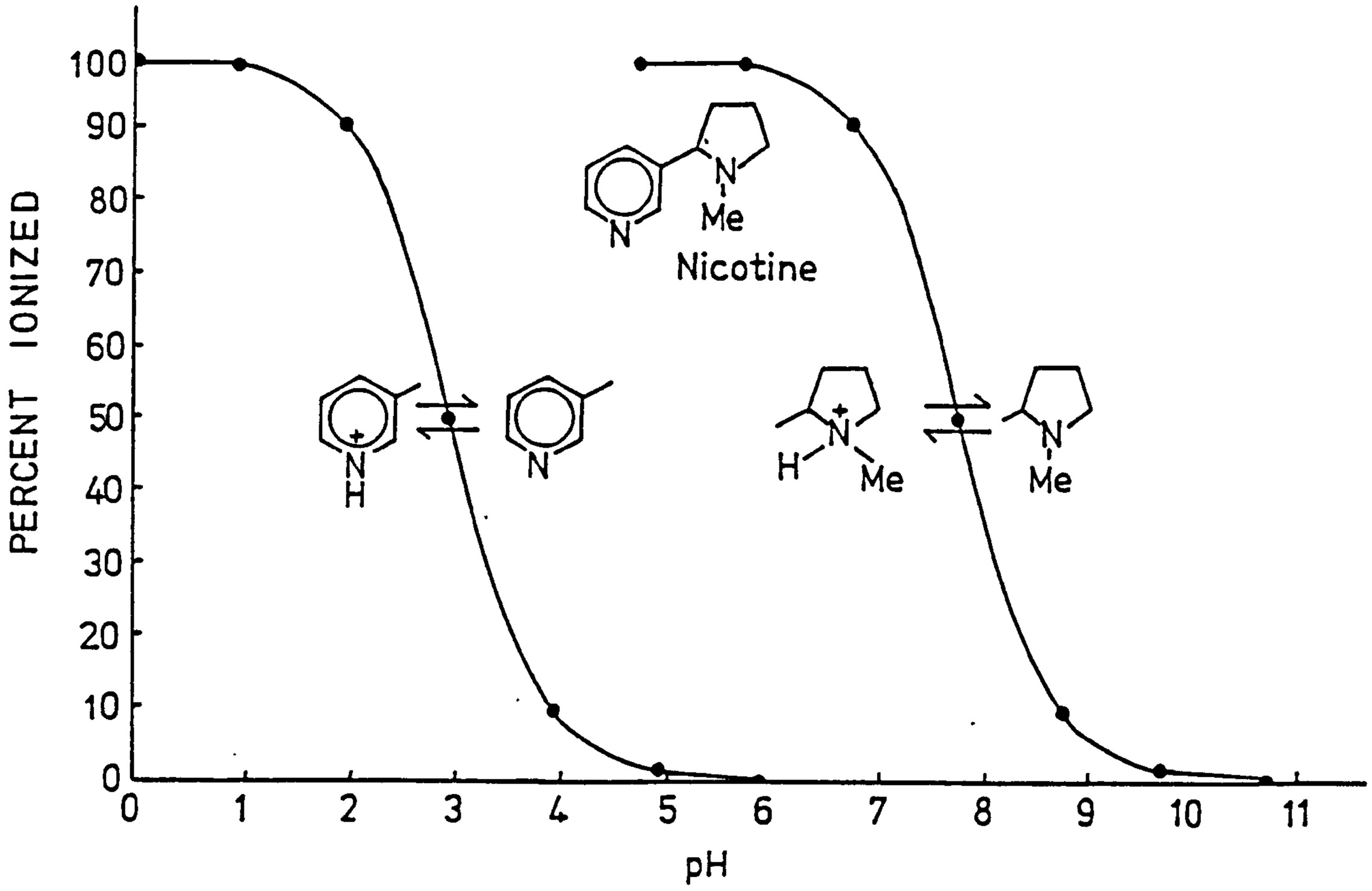
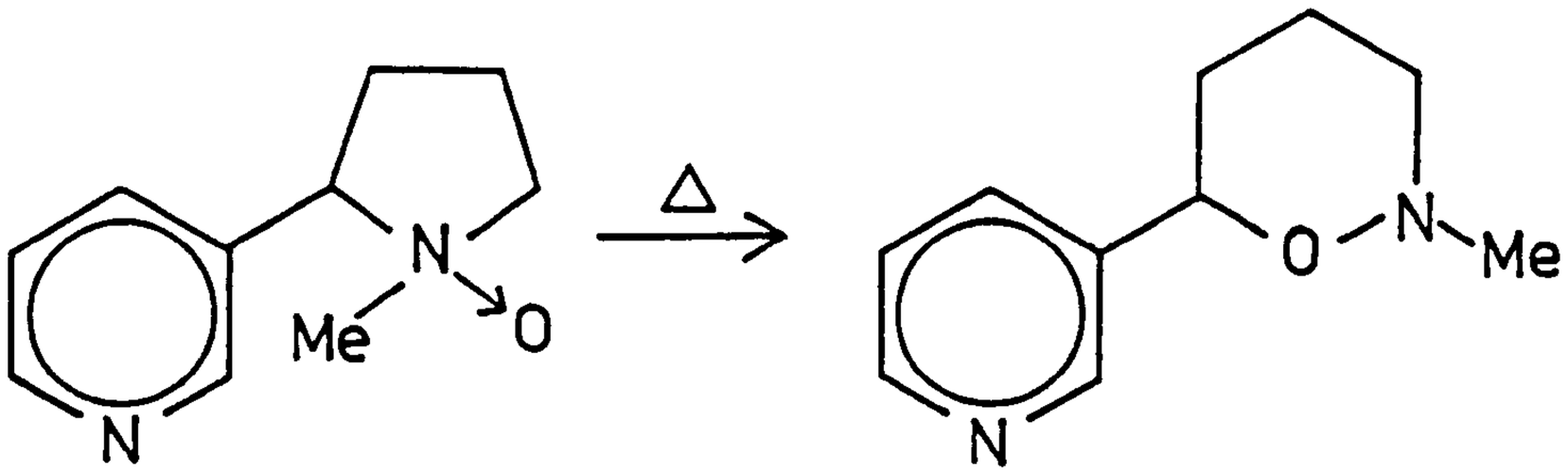


Figure 1.4: Calculated dissociation curves for (-)-nicotine[142]

Cotinine, (S)-5-(3'-pyridyl)-1-methylpyrrolidone-2, ($C_{10}H_{12}N_2O$), is a white (when pure), hygroscopic, crystalline solid, melting point 41-43°C, which turns brown on prolonged exposure to the atmosphere. Aqueous solutions of cotinine are found to be only mildly basic due to conjugation of the pyrrole nitrogen lone pair with the sp^2 hybridized carbon of the carbonyl group. The pK_a values are 2.7 and 4.5 for the pyridyl and the pyrrolidyl nitrogens respectively. At pH 7.4 only 0.1% monoprotonated cotinine exists, the remaining 99.9% being unprotonated. Cotinine is much more polar than nicotine due to the presence of the carbonyl group, and is soluble in water, dichloromethane and hexane but insoluble in ether.

Nicotine-1'-N-oxide, $C_{10}H_{14}N_2O$, is a white crystalline compound, soluble in cold water and chloroform but insoluble in ether. Like

cotinine, it is hygroscopic and must be stored in a dry atmosphere. Above its melting point (~ 170-171°C), it undergoes a transformation into an isomeric base which has been proved to be 2-methyl-6-pyridyl-(3') tetrahydro-1,2-oxazine.

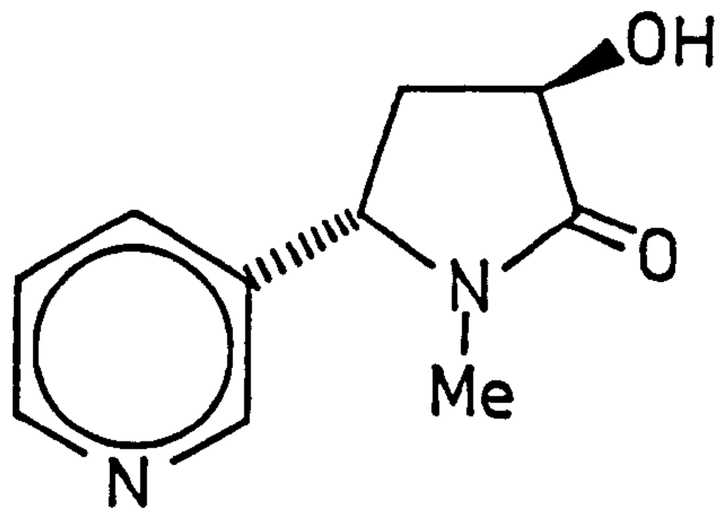


Nicotine-1'-N-oxide has been resolved into two optically active stereoisomers, both of which have been identified in the urine of cigarette smokers.[64] As optically pure (-)-nicotine is produced by the tobacco plant, the configuration at the asymmetric carbon atom of the pyrrolidine ring is fixed. The existence of the two stereoisomers is due to different arrangements of the groups at the quaternary nitrogen atom of the pyrrolidine ring, where one isomer has the pyridyl and methyl groups cis to each other, the other isomer having the pyridyl group and the N-oxide oxygen in the cis arrangement, as shown below.

where R = 3-pyridyl

The two stereoisomers have been labelled cis and trans although structures have not been assigned. Steric hindrance would exist between the pyridyl and methyl groups when they are cis to each other suggesting that this may be the form which is laevorotatory as it is the more unstable of the two. As the stereoisomers are not enantiomorphs, they do not have equal and opposite optical activities, however one is dextrorotatory, $[\alpha]_D^{22}$ + 65.1, and the other laevorotatory, $[\alpha]_D^{22}$ - 76.0.[64]

3' Hydroxycotinine, ($C_{10}H_{12}N_2O_2$), is a cream, hygroscopic, crystalline compound, melting point 110-111°C. Again, more than one isomer exists, however only trans-3'-hydroxycotinine has been identified in smokers' urine.[185] The structure of the isomer is shown below, with the pyridyl and hydroxyl groups in a trans arrangement.



1.22 Analytical Techniques which have been applied to the Analysis of Nicotine and its Metabolites

In early studies where the contribution made by different organs to the metabolism of nicotine had to be established and the identity of the metabolites formed was being sought, the analytical methods used were mainly qualitative. Many studies involved the use of ^{14}C-nicotine. The radioactive compounds were separated by paper or thin layer chromatography (TLC).[48] TLC is a good qualitative method but it has quantitative limitations in general use.

As the health risks involved in smoking began to emerge, it was thought that a quantitative method for the analysis of nicotine and its metabolites in body fluids would provide useful information on the exposure of the smoker to nicotine and other related compounds.

The nicotine doses used in many of the early experiments were far in excess of the quantities attainable by smoking. The method for the determination of nicotine and its metabolites should be capable not only of dealing with tobacco smokers who may receive several milligrams of nicotine per day, but also the non-smokers who may ingest only trace quantities of the alkaloid from a smoky room or non-tobacco sources. More sensitive and specific methods were required, with the potential for use in routine analysis.

1.22.1 GC Methods

Gas chromatography had been used to detect and quantitate nicotine and other alkaloids in tobacco plants and cigarette smoke.[143-145] Analytical techniques applied to the quantitation of nicotine in biological systems also include GC. McNiven et al. (1965)[146] used GC to determine nicotine and its metabolite cotinine in smokers' urine. The success of gas chromatography is dependent on the extraction/clean up procedure, the component separation and the detector characteristics.[147]

Due to the nature of biological fluids, nicotine must be extracted before chromatographic analysis. Several published assays[89,90,148] are in fact very time-consuming, involving a large number of extraction steps, in the method of Burrows et al.[149] a steam distillation is required, or the sequential extraction of nicotine and cotinine.[150,151] This renders them unsuitable for routine analysis of large numbers of clinical samples. Also, the extraction procedure should be simple to avoid sample contamination by extraneous nicotine. Some methods incorporate an evaporation step in order to obtain a concentrated sample.[148] This increases preparation time and also results in some loss of nicotine due to its volatility. Some form of prior concentration may be necessary, otherwise the system may have to operate close to its detection limit. Hill et al.[152] have used a solvent extraction method which concentrates the sample without evaporation.

The choice of an internal standard is a very important part of the development of an analytical method. Many of the methods for the determination of nicotine and its metabolites lack an internal

standard.[89,153,154] In several others a poor choice of internal standard has been included. Feyerabend et al.[155] selected quinoline but Hengen and Hengen[150] were unable to obtain consistent results using this method. Jacob et al.[151] also found large variation in peak height ratios of nicotine to quinoline even for the same ether extract. They attributed this to the selective loss of nicotine on the glassware or in the GC as nicotine is more basic than quinoline ($pK_a \sim 8$ and $pK_a \sim 4$ respectively).

Ideally the IS should be added directly to the sample to monitor extraction losses. The differences in solvent partitioning and chromatographic properties of nicotine and cotinine have led to the use of separate internal standards.[156] Jacob et al.[151] used two internal standards, N-(2-methoxyethyl)-norcotinine to monitor cotinine and N-ethylnornicotine to monitor nicotine. N-(2-methoxyethyl) nornicotine has similar solvent partitioning properties and pK_a value and is a structural analogue of cotinine, unlike lidocaine used in many published methods[150,157] which is more lipophilic and more basic than cotinine. Structural analogues have the added advantage of having retention times and detector responses which are close to those of the components of interest and so improve precision of the chromatographic analysis.

Even after the extraction and clean up procedures, a relatively complex mixture of compounds remains, giving many peaks in the GC. In fact, Gruenke et al.[158] found GC-FID inadequate when applied to the analysis of smokers' and non-smokers' plasma. The complexity of the GC spectra can be substantially reduced by the introduction of a nitrogen sensitive alkali tip detector which minimizes interference

as it does not respond to organic compounds containing only carbon, hydrogen and oxygen atoms. The nitrogen sensitive alkali tip detector is also more sensitive than the FID. Although it is based on the FID, the incorporation of the alkali bead or ring enhances the detector response to compounds containing a heteratom, the choice of alkali metal salt determines which heteratom. The response may be enhanced by as much as a factor of 1000. Nitrogen sensitive detectors have been criticised for their low long-term stability. Stehlik et al.[157] considered this a serious drawback for routine applications.

The introduction of capillary columns provides a much higher separation efficiency than packed columns, narrower peak shapes, higher resolution and reproducible retention times.[159] The use of both capillary GC and nitrogen sensitive detectors allow multistage extraction and clean up procedures to be avoided while providing good sensitivity and specificity.[168] Using a small number of steps in the extraction process means that the loss in separation must be compensated for by more efficient GC and/or higher detector sensitivity.

Capillary GC has also been combined with high resolution mass spectrometry.[161] This is a very sensitive, selective and rapid technique for the quantitative analysis of nicotine in biological fluids. However, the high capital investment required and the complexity of the instrument make it an unlikely choice for routine analysis.[161]

The significant and variable adsorption of nicotine, particularly at low concentrations, makes reliable results difficult to obtain.[162] A suitable internal standard enables such losses to be accounted for. Also adsorption can be suppressed by the addition of NH_4OH

during sample preparation, as suggested by Grubner et al.[154] Hill et al.[152] found it necessary to silanize all glassware with dimethyldichlorosilane initially. The handling of samples would be simplified if adsorption properties could be reduced in a derivatization step. Hartvig et al.[162] ingeniously achieved not only the formation of a non-adsorptive derivative of nicotine but one with excellent electron capture detection sensitivity (picogram levels), namely the δ-trichloroethyl carbamate of nicotine. Also once the derivative has been formed, the influence from environmental nicotine is unimportant.

Many methods of analysis quantitate both nicotine and cotinine concurrently, by GC, at different column temperatures.[150,147,151] Temperature programming can be used for the simultaneous analysis of nicotine and cotinine,[163] however some workers[164] thought it prevented rapid analysis. On packed columns cotinine requires a high elution temperature and, as GC phases are labile, this limits the lifetime of the column considerably. Capillary columns are much superior in this respect.

1.22.2 Radioimmunoassays

Radioimmunoassay is another technique which has been applied to analysis.[97,165] The initial development of a satisfactory radioimmunoassay may require several months but once developed the technique is simple to perform, requiring minimal sample preparation, and can be used to analyse large numbers of samples. These methods are very sensitive. Langone et al.[165] developed a method for the estimation of nicotine and cotinine at the picomole level in the presence of each other and other known metabolites. However, the specific antibodies

are not readily available, making chromatographic techniques more generally accessible and less expensive.

1.22.3 HPLC Methods

The remaining published methods for the analysis of nicotine and its metabolites have been carried out using HPLC. Hill et al.[152] considered HPLC had insufficient sensitivity for trace analysis. Watson and Maskarinec et al.[166] have developed methods for the estimation of nicotine and cotinine by HPLC with UV detection. Jane[167] has described the separation of nicotine from common drugs of abuse, again using isocratic HPLC and UV detection.

Although many different methods of analysis have been proposed for the determination of nicotine and its main metabolite cotinine, very few methods have attempted to determine any of the other numerous metabolites. N-Oxides in general and nicotine-1'-N-oxide, being thermally labile, cannot be analysed directly using GC because of the high temperatures involved. Analysis has been carried out by the extraction/quantitation of unmetabolized nicotine in the sample followed by reduction of nicotine-1'-N-oxide to nicotine with $TiCl_3$ and then assayed for nicotine as before.[82]

Direct analysis of nicotine-1'-N-oxide is possible by using HPLC. Kyerematen et al.[168] successfully determined nicotine and its principal metabolites cotinine and nicotine-1'-N-oxide in plasma and urine. Both UV detection and radiolabelling plus a liquid scintillation counter were used. The use of ^{14}C-labelled nicotine is confined to certain research purposes only and does not solve the problem of routine analysis of human biological fluids.

Thompson et al.[169] carried out nicotine-1'-N-oxide analysis in mouse tissue. Tissues are more complex biological matrices than biological fluids. UV detection was possible, but in a pharmacokinetic study, on the elimination phase of the profile, several tissue concentrations were below the detection limit. Again, it was necessary to use radiolabelling below ~ 30 ng/g, above this level accurate UV data was obtained. Horstmann's[170] HPLC/UV method for the determination of nicotine and cotinine allowed differentiation between smokers and non-smokers but he found that, in the majority of cases, non-smokers had nicotine and cotinine concentrations below the detection limit. The detection limit is dependent upon the UV extinction coefficient of the compound.[167]

N-methylated quaternary metabolites of nicotine are water soluble, highly polar and non-volatile. Without appropriate derivatization to a more volatile compound, GC determination is not possible. Cundy and Crooks[171] developed a HPLC method using RP-IIC and [2'-^{14}C]-nicotine with liquid scintillation counting for the detection and quantitation of the small amounts of metabolites present. Analysis of N-methylated metabolites has also been reported by Mousa et al.[172] In this case LC coupled with coulometric electrochemical detection has been used for the determination of both nicotine and N-methylnicotinium ion. The metabolites cotinine and nicotine-1'-N-oxide are not observed in the ED sensitivity range using the conditions employed for the nicotine analysis.

1.22.4 Sample Matrices and Other Considerations Prior to Analysis

Different samples have been used in the reported analytical methods; tissue, blood, plasma, saliva and urine. Urine and saliva have the advantage that they do not require invasive sampling methods. Also nicotine is present in larger amounts in the urine of smokers than in venous blood[149] and sample volume is not a restriction. The concentrations of nicotine and cotinine in most urine samples are below 1 µg/ml, whereas plasma usually has nicotine of less than 60 ng/ml and cotinine below 200 ng/ml.[157] The low concentration of the components of interest together with the small volumes of blood/plasma available demand a very sensitive method of analysis and a single determination of all compounds. Feyerabend et al.[155] described a micro method for the analysis of nicotine alone, requiring only 100 µl of sample. At very low concentrations sensitivity was increased by using 200 µl sample volumes.

Automation is also an important consideration when carrying out large numbers of routine analysis. Automation has stringent requirements; preferably a one step extraction procedure for the components of interest, simultaneous determination by the chosen analytical technique, high sensitivity and high instrument stability.

Many workers considered that the determination of cotinine offered several advantages over that of nicotine alone.[173] They considered cotinine with its much longer biological half-life as an indicator of long-term nicotine exposure. Nicotine which is rapidly metabolized can only provide information about recent exposure and its clearance from plasma makes determination difficult.

As already discussed, differences in the intake of nicotine,

efficiency of metabolism, pH and flow rate of urine etc. all cause variation in the concentrations of nicotine and cotinine, therefore measurement of nicotine and its major metabolite cotinine (∿ 20% of the intake) may not be sufficient to give the researcher the desired information on the smoking behaviour or exposure of the individual. A flexible method of analysis for nicotine and as many of its metabolites as possible is required. A summary of different methods of analysis has been included in Table 1.4.

1.23 Objectives

This research project aims to develop and evaluate a flexible HPLC method for the determination of nicotine and its two major metabolites cotinine and nicotine-1'-N-oxide. Another very important metabolite, 3' hydroxycotinine, was also included at a later stage in the development. The importance of 3' hydroxycotinine has perhaps been underestimated, as Neurath[174] has reported this metabolite to be present in amounts second only to cotinine. UV is the chosen method of detection although there has been some doubt about its sensitivity at low concentrations. It has high instrumental stability, especially important if automation is to be a consideration. In an attempt to overcome any doubts about the sensitivity, urine is the most suitable sample matrix where the concentrations of the parent compound and its metabolites were reported to be highest and sample volume is not a constraining factor. From earlier work it appears that all metabolites (except CO_2) are excreted in the urine and sample collection has the advantage of being non-invasive. Also, partially

Table 1.4

METHODS OF ANALYSIS FOR THE DETERMINATION OF NICOTINE, COTININE, NICOTINE-1'-N-OXIDE AND 3' HYDROXYCOTININE IN BIOLOGICAL SAMPLES

METHOD/DETECTION	SAMPLE/SENSITIVITY	COMPOUNDS ANALYSED	INTERNAL STANDARD	REF.
GC-strontium 90/ argon ionization detector	Urine (5 ml)	Nicotine	3-methyl, 3-phenyl piperidine (external standard)	146
GC-FID	Urine (5 ml) → 50 µl dichloromethane or ether	Nicotine and cotinine separately	Nic. IS:- chlorphentermine Cot. IS:- lignocaine	175
GC-FID	Whole blood (10 ml) Detection limit for nicotine: 1ng	Nicotine	Quinoline	149
GLC-AFID	Blood (10 ml) Sensitivity 1 ng ml^{-1} of nicotine in a 2.5 ml sample	Nicotine	Modaline	86
Radio-immunoassay	Serum and urine 350 pg - detection limit	Nicotine and cotinine	-	165
Radio-immunoassay	Serum (smokers') (0.1 ml)	Cotinine and nicotine	-	173
GC-FID	Urine (20 ml) Final volume 0.5 ml chloroform	Nicotine and cotinine separately	no IS	153
GC-AFID	Plasma (3 ml) → n-heptane (50 µl)	Nicotine	no IS	89
Radiochemical	Blood (10 ml)	Nicotine and cotinine	no IS	131
HPLC	----	Nicotine	-	167
HPLC isocratic	Urine (3 ml) Minimum detectable level 5 ng on column	Nicotine and cotinine simultaneously	desmethylimipramine (HPLC) Quinoline (GC)	164
GLC-AFID	Plasma (1 ml) Sensitivity 0.1 µg l^{-1}	Nicotine and cotinine separately	Nic. IS:- modaline Cot. IS:- lidocaine	150
Cap GC-MS (SIM mode)	Plasma (3 ml) → benzene 100 µl Lower limit of detection 5 ng ml^{-1}	Nicotine	Quinoline	148
HPLC/UV Cap GC/AFID	Urine (20 ml), plasma (1-5 ml) Detection limit: 2 ng ml^{-1} urine	Nicotine and cotinine simultaneously (nornicotine)	desmethylimipramine	166
GC-MS (SIM)	Not included	Nicotine	deuterated nicotine 5',5'-d_2	158
GC-ECD and AFID	Plasma ($\leq$ 1 ml) Limit of quantitation: 10 ng ml^{-1} in plasma	Nicotine	N-n-propylnornicotine	162
GC-AFID	Sample: plasma, urine, saliva or breast milk. 3 ml → butyl acetate (50 µl), 100 µl → di-isopropyl ether (5 µl)	Nicotine	Quinoline	155
"	"	"	Isoquinoline	176
GC-AFID	Sample (1.0 ml) → Acetone (50 µl) Lower limit of detection 1 ng ml^{-1}	Cotinine	Pheniramine maleate	177
GC-AFID	Blood plasma (0.4 ml) Lowest detectable amount 2-5 pg (nicotine)	Nicotine	no IS	154
GC-AFID (temp. programming)	Plasma (1 ml) Sensitive to 2 ng nicotine per ml extracted and 5 ng cotinine per ml extracted	Nicotine and cotinine simultaneously	Ketamine	163

Table 1.4 (continued)

METHOD/DETECTION	SAMPLE/SENSITIVITY	COMPOUNDS ANALYSED	INTERNAL STANDARD	REF.
GC-AFID	Blood, plasma or urine (1 ml)	Nicotine and cotinine separately	Nic. IS:- N-ethylnor-nicotine Cot. IS:- N-(2-methoxy-ethyl)-norcotinine	151
HPLC/UV and Radiometric HPLC assay	Plasma or urine (2 ml) Sensitivity: 60 dpm for radio-metric assay; 5 ng for unlabelled compounds	Nicotine, cotinine and Nicotine-1'-N-oxide	Acetanilid	168
GC-AFID	Plasma (0.5 ml) → isoamyl alcohol (60 µl) Sensitivity: nicotine 1 ng ml^{-1} cotinine 5 ng ml^{-1}	Nicotine and cotinine simultaneously	Ketamine	178
GC-MS (SIM)	Plasma (1 ml) Lower limit of detection 5 ng ml^{-1}	Nicotine	(5'5'-$^2H_2$) nicotine	161
Cap GC-AFID and GC-MS	Tissue (1 g) Sensitivity 2-3 ng per g of tissue for both compounds	Nicotine and cotinine separately	Cot.d_3 -1'-trideuteromethyl norcotinine Nic.d_3 - 1'-trideuteromethyl nornicotine for mass spec. work; Methylanabasine and 1-methyl-6-(3-pyridyl)-2-piperidone for GC work	159
Cap GC-FID Temp. programmed	Urine (4 ml), plasma (2 ml) minimum detectable concentration 0.03 µg ml^{-1} nicotine and cotinine, 1.0 µg ml^{-1}, both N-oxides — in urine 5 ng ml^{-1}, nicotine and cotinine, 15 ng ml^{-1}, both N-oxides — in plasma	Nicotine and cotinine simultaneously. Also Nicotine-1'-N-oxide and cotinine-1'-N-oxide	Phendimetrazine-N-oxide Quinoline Lidocaine Phendimetrazine	157
Cap GC-AFID	Plasma (1.0 ml and 0.1 ml) Detection limit: nicotine 5 pg, cotinine 20 pg Minimum detectable amounts per ml of plasma: nicotine 0.1 ng ml^{-1}, cotinine 0.4 ng ml^{-1}	Nicotine and cotinine	Nic. IS:- N-methyl-anabasine Cot. IS:- N-ethylnor-cotinine	147
GC thermionic detection	Plasma (2.0 ml) Determinations possible to 5 ng ml^{-1}	Nicotine	Modaline or N-ethylnornicotine	152
HPLC/UV detection, λ = 257 nm	Plasma and saliva (1 ml)	Cotinine	2-phenylimidazole	179
HPLC-electrochemical detection	----	Nicotine and N-methyl nicotinium ion	-	172
GC-FID	Urine samples (25 ml) It is possible to measure 50 ng ml^{-1}	Nicotine and cotinine separately at two different temperatures	no IS	180
HPLC/UV and radiometric detection	Tissue homogenate (1 g) Limit of detection for NNO UV 20-25 ng EC 25-30 ng	Nicotine-1'-N-oxide only. Separation of Nicotine, Cotinine, NNO and CNO given	no IS	169
Cap GC-MS (SIM)	Plasma and urine (0.25-1.0 ml of sample) Limit of detection: < 1 ng ml^{-1} cotinine, using plasma (1 ml) or urine (0.25 ml)	Cotinine	Tri-deuterated cotinine (3-[$^3H_3$]methyl cotinine)	160

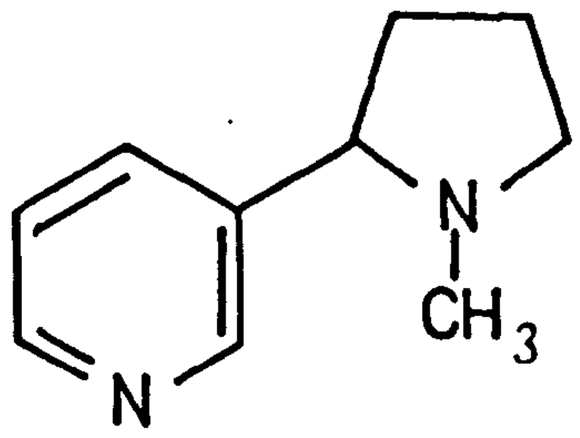
Table 1.4 (continued)

METHOD/DETECTION	SAMPLE/SENSITIVITY	COMPOUNDS ANALYSED	INTERNAL STANDARD	REF.
HPLC/UV	Plasma (0.5 ml) No sensitivity levels quoted	Nicotine and cotinine simultaneously	no IS	181
Cap GC-NPID Temperature programmed	Urine samples (20 ml)	Nicotine and cotinine	n-undecane nitrile	182
HPLC/UV λ = 260 nm	Urine (4.0 ml) Minimum detectable amounts of nicotine and cotinine ~ 15 ng Quantitation of peaks > 50 ng ml^{-1} of the alkaloid	Nicotine and cotinine	Amphetamine	170
GC-AFID Temperature programmed	Plasma samples (1.0 ml) Nicotine and cotinine >1 ng ml^{-1} have been detected	Nicotine and cotinine simultaneously	Nic. IS:- N-ethylnor-nicotine Cot. IS:- N-ethylnor-cotinine	183
Cap GC/ thermionic N specific detection	Urine, saliva and plasma (0.1-1.0 ml) Detection limit of 0.1 ng ml^{-1} of body fluid for nicotine and cotinine	Nicotine and cotinine separately at two different column temperatures	Nic. IS:- N-methyl-anabasine Cot. IS:- N-ethylnor-cotinine	156
GC-MS	Plasma (200 µl) Detection limit: 100 nmol l^{-1}	Cotinine	Methylprylone (Noludar)	184
GC-ECD	Plasma or urine (1 ml) Limits of quantitation and detection are 5 ng ml^{-1} and 1 ng ml^{-1} respectively	Trans 3' Hydroxy-cotinine	No IS	185
HPLC/UV detection, also radiometric detection	Urine (20-50 µl)	N-methylated metabolites, cotinine, nornicotine, nicotine, nicotine-1'-N-oxide	No IS	171

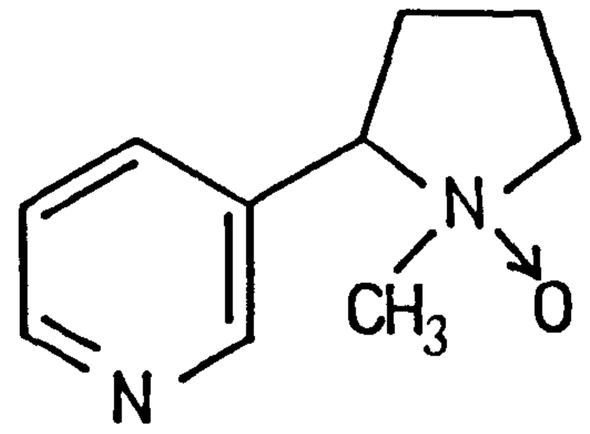
or wholly aqueous samples are compatible with HPLC analysis.

HPLC has the potential to analyse for nicotine, cotinine, nicotine-1'-N-oxide and 3' hydroxycotinine simultaneously, quantitatively and without derivatization, structures shown in figure 1.5.

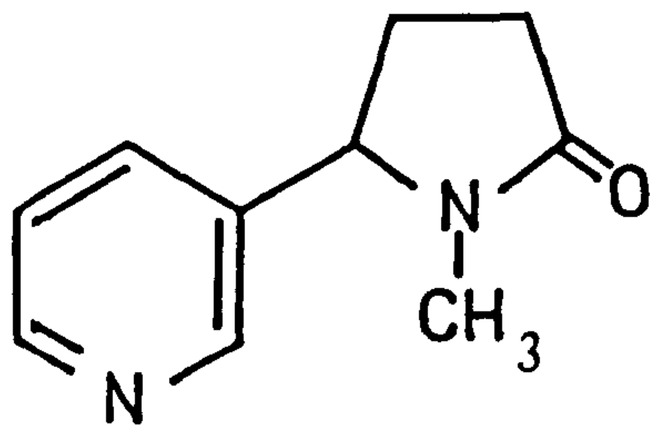
Aspects of HPLC relevant to the separation of nicotine and its metabolites will be discussed in Chapter 2.



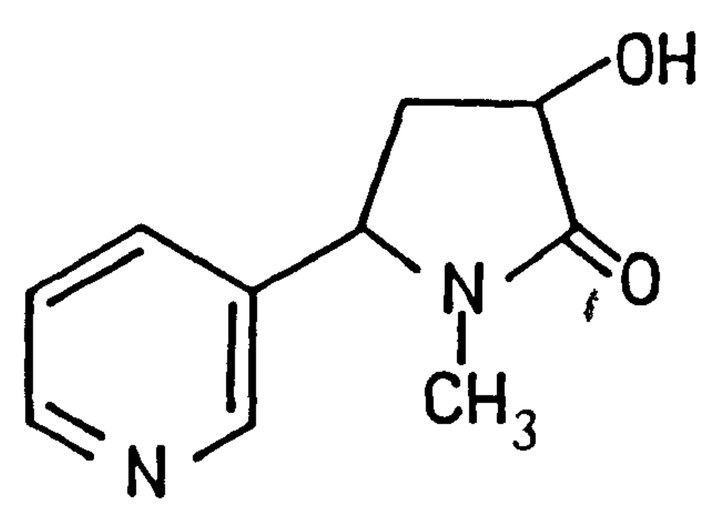
Nicotine



Nicotine-1´-N-Oxide



Cotinine



3´ Hydroxycotinine

Figure 1.5: Nicotine and its metabolites of interest in this study

CHAPTER 2

DEVELOPMENT AND OPTIMIZATION OF AN HPLC METHOD FOR THE ANALYSIS OF NICOTINE AND ITS METABOLITES

2.1 Introduction

The term chromatography covers a variety of separation methods which use two mutually immiscible phases, one stationary and the other mobile. The sample to be separated is introduced into the mobile phase and as it is carried through the system in the mobile phase it undergoes a series of interactions with both the mobile and the stationary phases. In order to achieve a separation the differences in the physical or chemical properties of the components in the sample are exploited. The more interaction with the stationary phase the longer the retention time. Separation results when the interactions of one component with the stationary phase are such that it is eluted without overlap with any other component in the sample. The stationary phase can be either a solid or a liquid, the mobile phase a liquid or a gas.

In high performance liquid chromatography, HPLC, the stationary phase is held in a rigid column, usually constructed of stainless steel, and the liquid mobile phase is forced through under pressure. Liquid chromatography may often achieve separations that are impossible by gas chromatography, GC, where the mobile phase is a gas. This is due to the use of low temperatures in HPLC. GC uses high temperatures and therefore is restricted to compounds which are sufficiently volatile and thermally stable. Also, there are two competing phases (mobile and stationary) in HPLC compared with one (the stationary phase) in GC. Furthermore, there is a wide choice of detectors available for use in LC, many of which are selective so making a complete separation on the column unnecessary, as a detector can be chosen to monitor only the species of interest.

The choice of a liquid chromatographic system to achieve a particular separation must be confirmed experimentally. Although the modes or mechanisms of chromatography will be considered individually, in practice a combination of mechanisms may be responsible for effecting a separation. Despite being desirable and very useful in achieving the end result - separation of a mixture - it also makes the prediction of retention behaviour more difficult.

2.2 HPLC Retention Mechanisms[186,187,188]

There are four basic LC mechanisms: liquid-liquid, liquid-solid, ion-exchange and size exclusion chromatography.

2.2.1 Partition Chromatography

In liquid-liquid or partition chromatography the components are partitioned between the immiscible liquids, a liquid mobile phase and an organic liquid stationary phase which is either coated onto a finely divided inert support or chemically bonded to the support material. The support surface should not interact with the sample molecules if true partition is desired. A balance between the attraction of mobile phase solvent and the stationary liquid phase for the sample, i.e. the solubility of sample components in the stationary and mobile phases, must be established. The polarity of the sample and the stationary phase may be matched and used with a mobile phase solvent of different polarity. The partitioning of the sample components between the mobile and stationary phases leads

to a differential rate of migration and separation of the components results.

The packing materials used in liquid-liquid chromatography can be divided into two types: normal phase and reverse phase. Normal phase chromatography refers to a system where the stationary phase is more polar than the mobile phase, whereas if the stationary phase is less polar than the mobile phase the process is termed reverse phase chromatography. The reverse phase mode is now the most widely used owing to the success of bonded stationary phases.

Reverse phase chromatography uses a hydrophobic bonded phase packing composed of an alkylsilyl bonded silica, e.g. octadecylsilane (ODS), (see figure 2.1). The octadecyl, (C_{18}), linear hydrocarbon chain is the most popular with C_8 and C_2 functional groups also

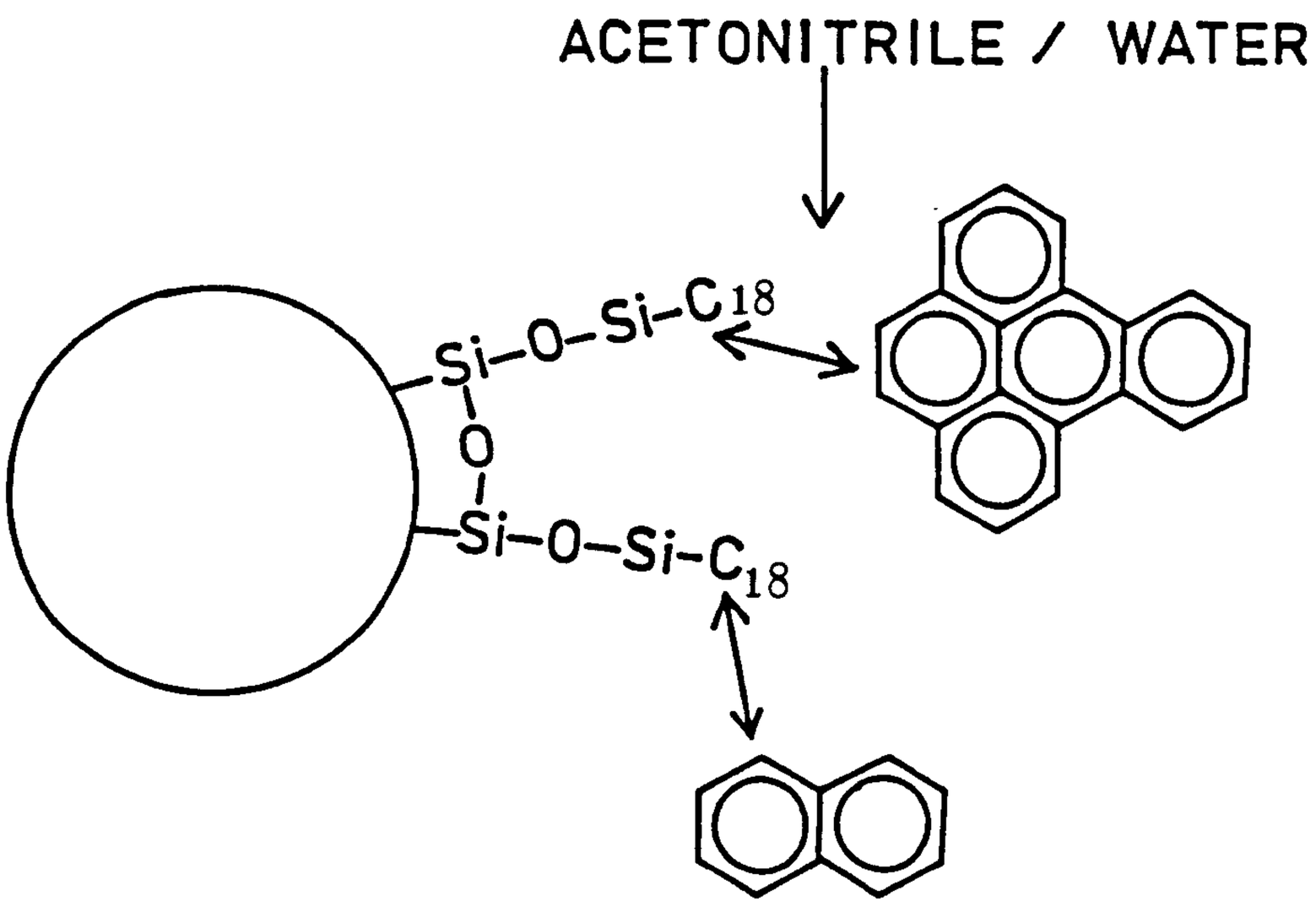


Figure 2.1: Reverse Phase Packing[189]

available. Typical mobile phases for reverse phase chromatography are aqueous buffers, methanol, acetonitrile and mixtures of water or buffers with these organic solvents. Polar substances prefer the mobile phase and therefore elute first with short retention times. As the hydrophobic character of the molecules increases retention also increases due to the mobile phase forcing the molecules to the hydrocarbonaceous bonded layer. The lower the polarity of the mobile phase, the higher is its eluent strength. Methanol and acetonitrile are the strongest eluents and water the weakest eluent employed in reverse phase chromatography. Intermediate strength eluents are obtained by mixing one of these solvents with water or an aqueous buffer.

Bonded phases can also possess functional groups, such as phenyl or cyano groups, incorporated on the saturated hydrocarbon chains which are chemically bonded to the support surface. Many different stationary phases are not necessary as the mobile phase composition can be used to alter the selectivity.

2.2.2 Ion Suppression Chromatography

The separation of samples which ionize in aqueous solution is possible on a reverse phase column by adjusting the pH of the eluent to suppress the ionization. Control of the pH usually requires a buffer in the mobile phase. Ion suppression is only applicable to weak acids and bases as pH must be kept within the range pH 3.0-8.0. Above pH 8 the silica support dissolves and below pH 3 the chemically bonded phase is irreversibly hydrolysed from the silica surface.

When the ionization of a weak acid is suppressed, i.e. at pH

values less than its pK_a, its retention time is increased.

$$AH + H_2O \rightleftharpoons A^- + H_3O^+$$

At pH values higher than its pK_a, it elutes faster. The ionic form of a sample elutes quickly compared to the neutral form because the ionic form is more soluble in the aqueous eluent (see figure 2.2). Similarly, a basic sample will elute faster at pH values below its pK_a value and will be retained longer at pH values higher than its pK_a where its ionization is suppressed.

$$B + H_2O \rightleftharpoons BH^+ + OH^-$$

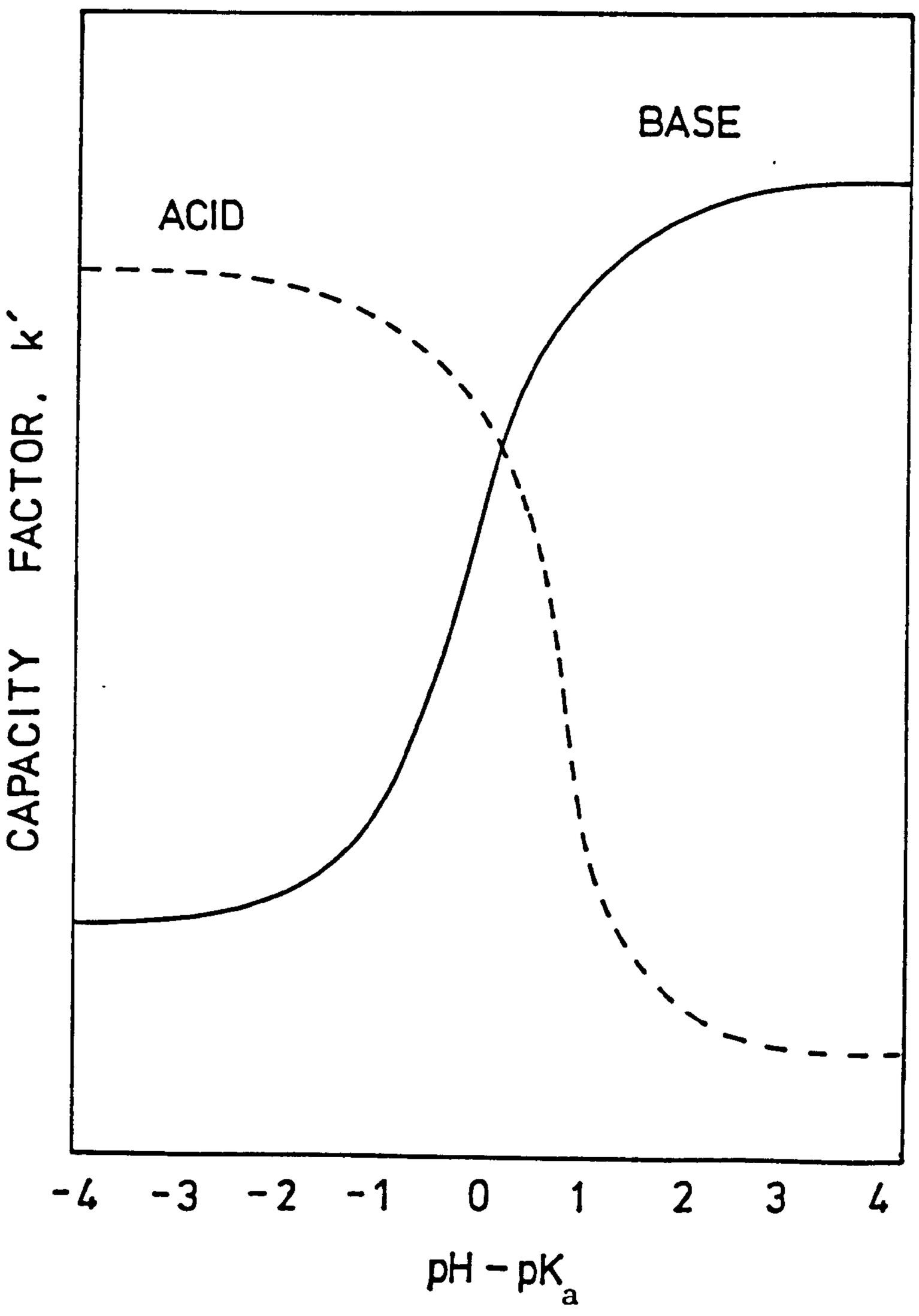


Figure 2.2: Effect of pH upon the retention of weak acids and bases on a non-polar stationary phase[190]

2.2.3 Ion-Interaction (or Ion-Pair) Chromatography

Due to the instability of bonded phases, the ion suppression method cannot deal with strong acids and bases. In the early 1960s, G. Schill et al.[191,192] applied ion-pair extractive techniques to modern liquid chromatography. The development of ion-interaction (or ion-pair) chromatography is generally attributed to them. By forming a coulombic association species between two ions of opposite charge using a suitable counterion, ionic or ionizable compounds can be partitioned into the non-polar stationary phase. The strength of the association between the ionized sample and the counterion affects the degree to which the retention is increased. There are many parameters that may play a significant role in reverse phase ion-interaction chromatography and these are summarized in table 2.1.

The mechanism which best describes reverse phase paired-ion chromatography is still being debated. There are three popular models. Both the ion-pair and the dynamic ion-exchange model describe the extreme situations. However, the third model, the ion-interaction model, takes into account the importance of both adsorptive and electrostatic attractions in governing retention in reverse phase paired-ion systems.

The ion-pair model states that ion-pairs form in the mobile phase prior to partition into the non-polar stationary phase. The more non-polar the 'ion-pair' complex, that is, the longer the alkyl chain on the counterion, the longer the retention, due to greater affinity for the stationary phase. This model is illustrated in figure 2.3.

The dynamic ion-exchange model states that the ion-pairing

TABLE 2.1: Adjustable Variables in RP-IIC [210]

Variable	Effect
Type of counterion	The better the ability to ion-pair, the longer the retention
Size of counterion	An increase in the size of the counterion will increase retention
Concentration of counterion	Increasing concentration increases retention up to a limit beyond which retention will decrease
pH	Effect is dependent upon nature of solute. Retention increases as pH maximises concentration of ionic form of solute
Type of organic modifier	Retention decreases with increasing lipophilic nature
Concentration of organic modifier	Retention decreases with increasing concentration
Temperature	Retention decreases as temperature increases
Stationary phase	More lipophilic or higher degree of coverage more retention

reagent, added to the mobile phase, is adsorbed onto the bonded alkyl stationary phase where it acts as adsorbed ion-exchange sites. The longer the alkyl chain length of the counterion, the greater the number of counterions adsorbed and hence the more ionic sites present for interaction with the ionic sample molecules, therefore the longer their retention. This model is illustrated in figure 2.4.

The third and final hypothesis, the ion-interaction model, states that the lipophilic reagent ions are adsorbed onto the stationary

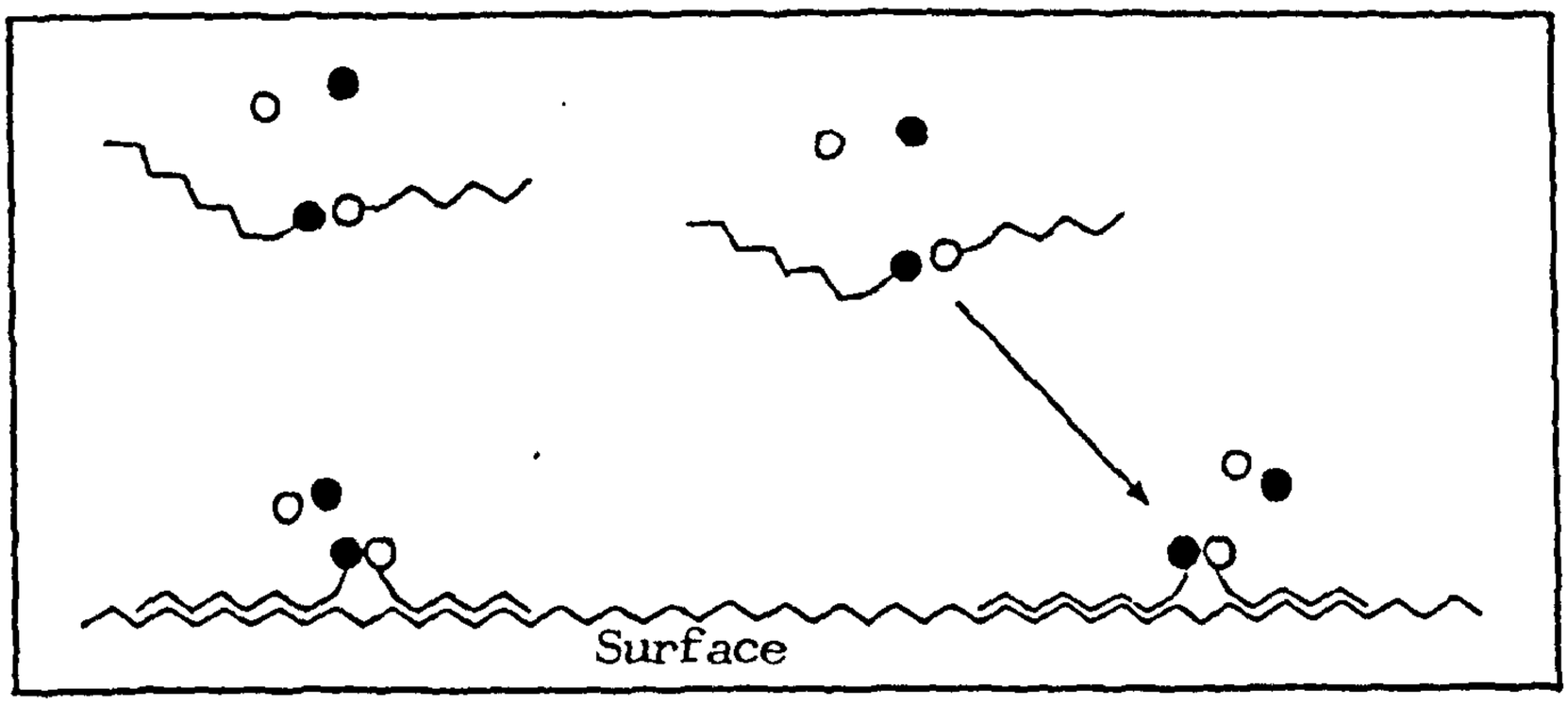


Figure 2.3: The Paired-Ion Model [193]

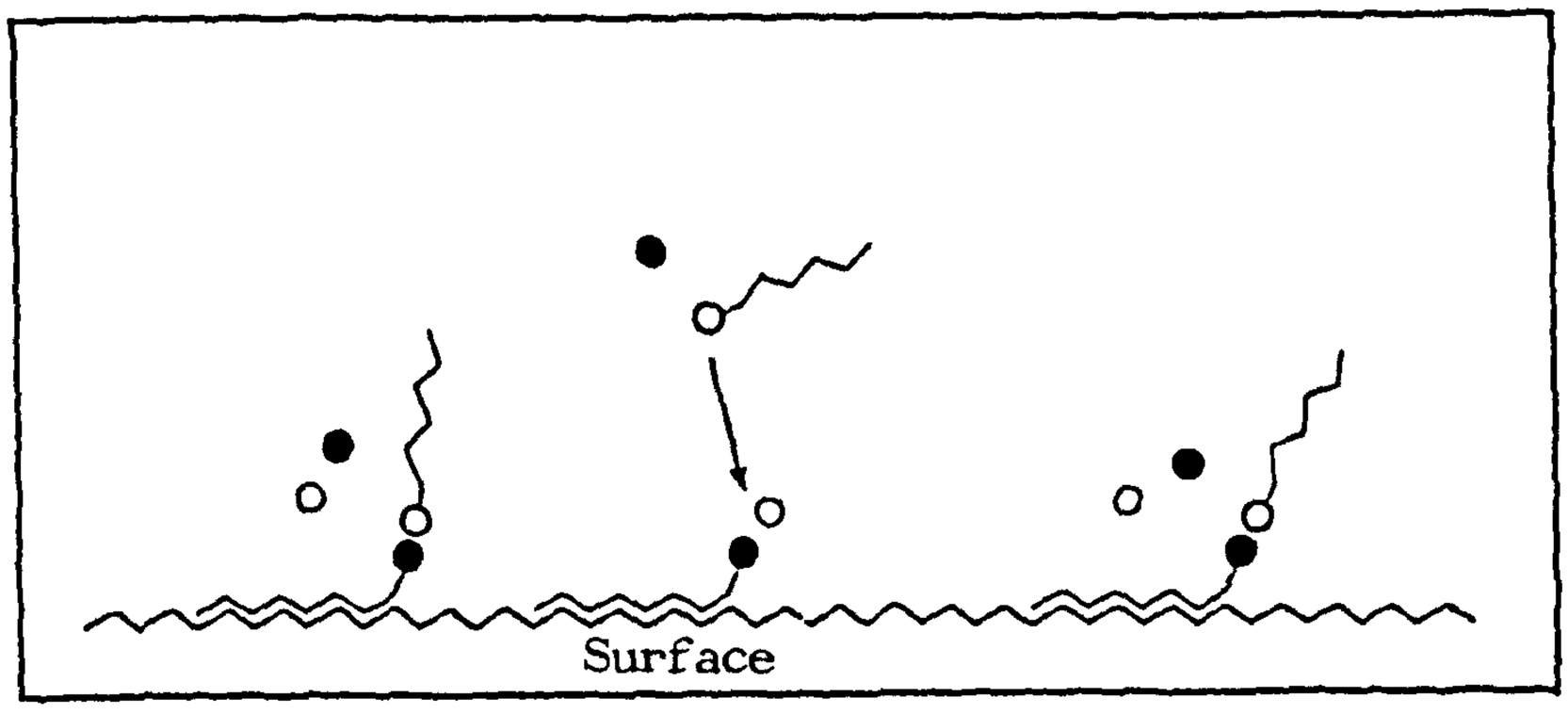


Figure 2.4: The Dynamic Ion-Exchange Model [193]

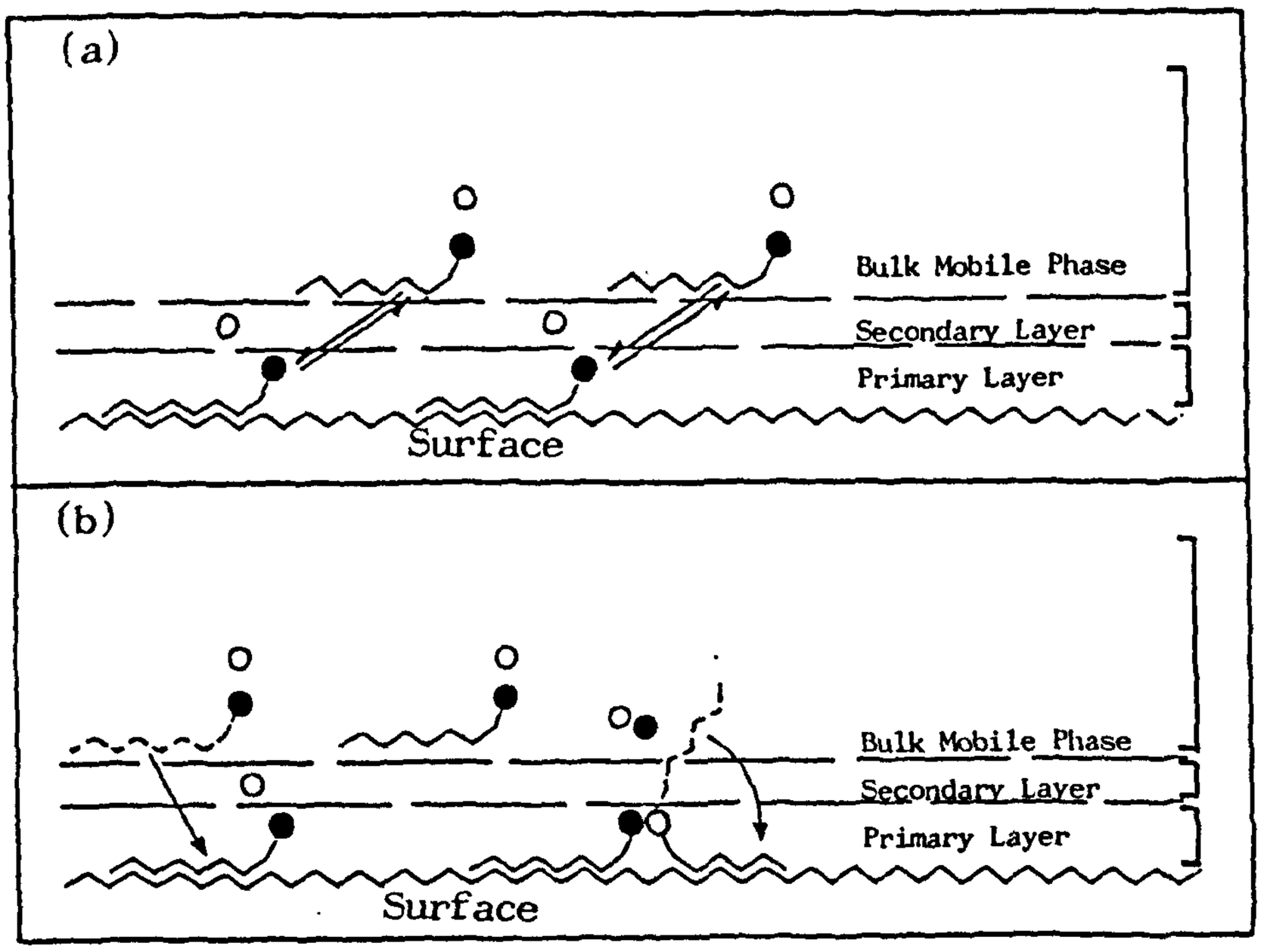


Figure 2.5: The Ion-Interaction Model: [193]
(a) Double Layer Formation, (b) Retention

Analyte

Ion-Interaction Agent

phase creating a charged primary ion layer and, consequently, an oppositely charged secondary layer. The retention of an ionized sample molecule results from electrostatic attraction to the primary layer of charged counterions and lipophilic attraction between the bonded hydrocarbon stationary phase and the carbon chain of the sample molecule. As an extra positive charge now exists in the primary layer, another counterion must also be adsorbed to maintain neutrality. Hence a pair of ions has been adsorbed. This two-stage model is illustrated in figures 2.5(a) and (b).

The exact mechanism may incorporate aspects of all three models but, whatever the true mechanism, ion-interaction chromatography results in some unique separations, allowing the separation of both ionized and unionized components simultaneously.

2.2.4 Adsorption Chromatography

In liquid-solid or adsorption chromatography physical surface forces are dominant. Components are separated on the basis of their polarities. With a polar stationary phase, such as silica gel, a relatively non-polar solvent, such as chloroform, will be used. Initially the adsorption sites are completely covered by solvent molecules. On introduction of the sample, the polar hydroxyl groups on the surface of the silica particles (Si-OH) interact with the functional groups of the sample (or solvent) molecules and, depending on the strength of these interactions, preferentially adsorb one molecule relative to another. These interactions are electrostatic, involving permanent dipoles or hydrogen bonding. The polarity of the mobile phase controls the desorption of the sample molecules

from the surface. The most polar molecules are strongly adsorbed (retained) on the silica surface (see figure 2.6). However, the more polar the mobile phase, the greater is its ability to displace the sample molecules from the surface and hence the shorter the retention time (or the lower the k' value).

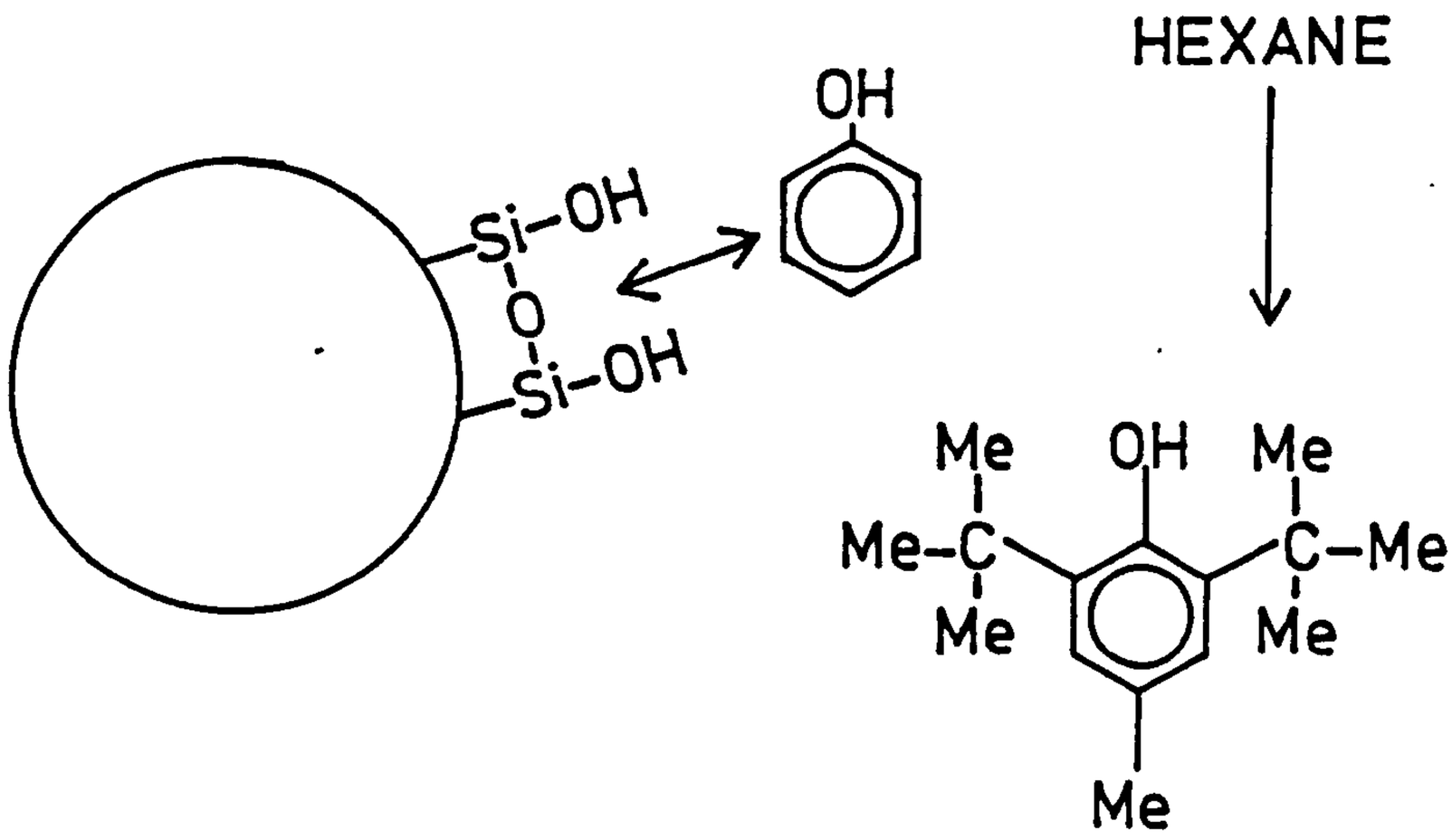


Figure 2.6: Normal Phase Packing[189]

2.2.5 Ion-Exchange Chromatography

Ion-exchange chromatography is used for compounds with ionic or ionizable functional groups. The stationary phase consists of two components: the polymer matrix with fixed ionic groups and attached counterions of opposite charge. These counterions can be exchanged for an equivalent number of other ions of the same sign (in the mobile phase). Separation depends on the charge-charge interactions between

the sample ions and the exchange site. Some ion exchangers bear positively charged groups and are used for exchanging anionic species, a process known as anion exchange. Exchanging cationic species, cation exchange, requires an ion-exchanger with negatively charged groups.

$$R^-Y^+ + X^+ \rightleftharpoons R^-X^+ + Y^+$$

2.2.6 Size Exclusion (or Gel Permeation) Chromatography

In size exclusion or gel permeation chromatography, separation is determined by the molecular size and shape of the components of the sample. It is a non-interactive mode of separation. The packing material is porous, with pores of a certain size. Molecules that are too large are excluded from all pores and therefore move through the column quickly whereas small molecules can penetrate most of the pores and so are retained by the packing material. The stationary phase can effect a separation according to molecular weight. Size exclusion (or gel permeation) chromatography is not applicable to the separation of nicotine and its metabolites.

2.3 Experimentally Relevant Chromatographic Theory[186,187,188]

2.3.1 Retention Parameters

The retention time, t_R, for a chromatographic peak is the time required for the sample to pass through the column, and is represented by

$$t_R = t_R' + t_M$$

where t_M represents the time spent in the void volume or the retention time of an 'unretained component' and t_R' is the time spent in the stationary phase. The unretained component is generally taken to be the injected solvent.

In some instances it is more convenient to work with retention volumes than with retention times. The retention volume, V_R, of any component may be obtained by measuring the retention time of the component in seconds and multiplying it by the volumetric flow rate in ml sec^{-1}, F,

$$V_R = t_R F \qquad \text{and} \qquad V_M = t_M F$$

V_M is a measure of the total volume of mobile phase contained within the column and it is often called the column 'dead' or 'void' volume.

Retention times or volumes for a set of components should be adjusted by subtracting t_M or V_M, as appropriate, before comparison.

$$V_R' = V_R - V_M \qquad \text{and} \qquad t_R' = t_R - t_M$$

2.3.2 Partition Ratio

The solute partition ratio, k', also called the capacity factor, column capacity ratio or the mass distribution ratio, is defined as the ratio of the total amount of a solute in the stationary phase to the amount in the mobile phase at equilibrium

$$k' = \frac{C_S V_S}{C_M V_M}$$

For a given system, k' is a measure of the time spent in the stationary phase relative to the time spent in the mobile phase, or the additional time (or volume) a solute band takes to elute over an unretained solute (for which k' = 0) divided by the elution time (or volume) of an unretained band:

$$k' = \frac{t_R - t_M}{t_M} = \frac{V_R - V_M}{V_M}$$

k' values greater than 8 waste valuable analytical time. Conversely, k' values less than unity are prone to interferences from unretained peaks and early peaks perhaps of little or no analytical interest.

2.3.3 Column Efficiency: Plate Number and Plate Height

The quantity N, the number of theoretical plates, is given by the equation

$$N = 16 \left[\frac{t_R'}{w_b} \right]^2$$

where $t_R' = t_R - t_M$ and w_b is the peak width at the base (the intersections of tangents to the inflection points with the base line), equal to 4σ in time units.

N is approximately constant for different bands in a chromatogram, for a particular set of operating conditions (the column, mobile phase, mobile phase velocity and temperature being fixed). N is a measure of column efficiency. As N remains constant for different bands in the chromatogram, the above equation predicts that the band width W_b increases as the band proceeds down the column or as t_R increases.

In comparing column efficiencies a more useful parameter is the height equivalent to a theoretical plate (HETP) or plate value H where

$$H = \frac{L}{N}$$

L is the length of the column and H measures the efficiency of the column per unit length. Small H values mean more efficient columns and large N values

$$H = \frac{L}{16}\left[\frac{w_b}{t'_R}\right]^2$$

The column efficiency can also be stated as a dimensionless quantity called the effective plate number, N_{eff}:

$$N_{eff} = \frac{L}{H} = 5.54\left[\frac{t'_R}{w_{\frac{1}{2}}}\right]^2$$

2.3.4 Band Broadening

A variety of factors cause broadening of a chromatographic peak. In the column, these include transverse and longitudinal diffusion in the mobile phase, the rate of equilibration of solute between the stationary and mobile phases (mass transfer), diffusion in the liquid stationary phase, and flow irregularities leading to convective mixing.

Eddy diffusion results from the inhomogeneity of flow velocities and path lengths, around the packing particles. Packing in the column is never perfect. Close to the column wall where the density of packing is comparatively low, some molecules will travel more rapidly (channelling) while other solute molecules will pass through the more tightly packed centre of the column at a lower velocity. Hence a distribution of solute velocities exists about the average velocity.

The contribution of eddy diffusion to the total plate height is represented by the equation

$$A = \lambda d_p$$

where d_p is the particle diameter and λ is an unspecified constant which is a function of the packing uniformity and the column geometry.

To minimize eddy diffusion d_p should be as small as possible. As d_p decreases, the inlet pressure required to force the mobile phase through the column increases. However, as column efficiency increases as d_p decreases, column length can be decreased, thus decreasing the required pressure drop.

High diffusion rates in the mobile phase cause solute bands to disperse axially. The contribution of longitudinal, or axial, diffusion to the plate height is given by

$$2\,\gamma D_M / \bar{u} = B / \bar{u}$$

where γ is an obstructive factor, D_M is the solute diffusion coefficient in the mobile phase and $\bar{u}$ is the average mobile phase velocity.

Longitudinal diffusion is particularly significant at low mobile phase velocities. As D_M in a gas phase is much larger (0.1-1 cm^2/sec) than it is in a liquid phase (1×10^{-5} cm^2/sec), the contribution of longitudinal diffusion to the overall plate height is much greater in GC than in LC.

The lack of instantaneous mass transfer at the solute/stationary phase interface makes a significant contribution to band broadening, and hence efficiency, and is proportional to

$$d_f^2 \bar{u}/D_s = C_{stationary}\,\bar{u}$$

where d_f is the thickness of the stationary phase and D_s is the diffusion coefficient of the solute in the stationary phase.

The rate of mass transfer can be improved by reducing the film thickness of the stationary phase, so reducing the distance that a solute molecule must diffuse within the stationary phase, see figure 2.7. Liquid layers should be as thin as possible without introducing adsorption effects on the support material. Also D_s is smaller the more viscous the liquid stationary phase, although it increases as the temperature rises.

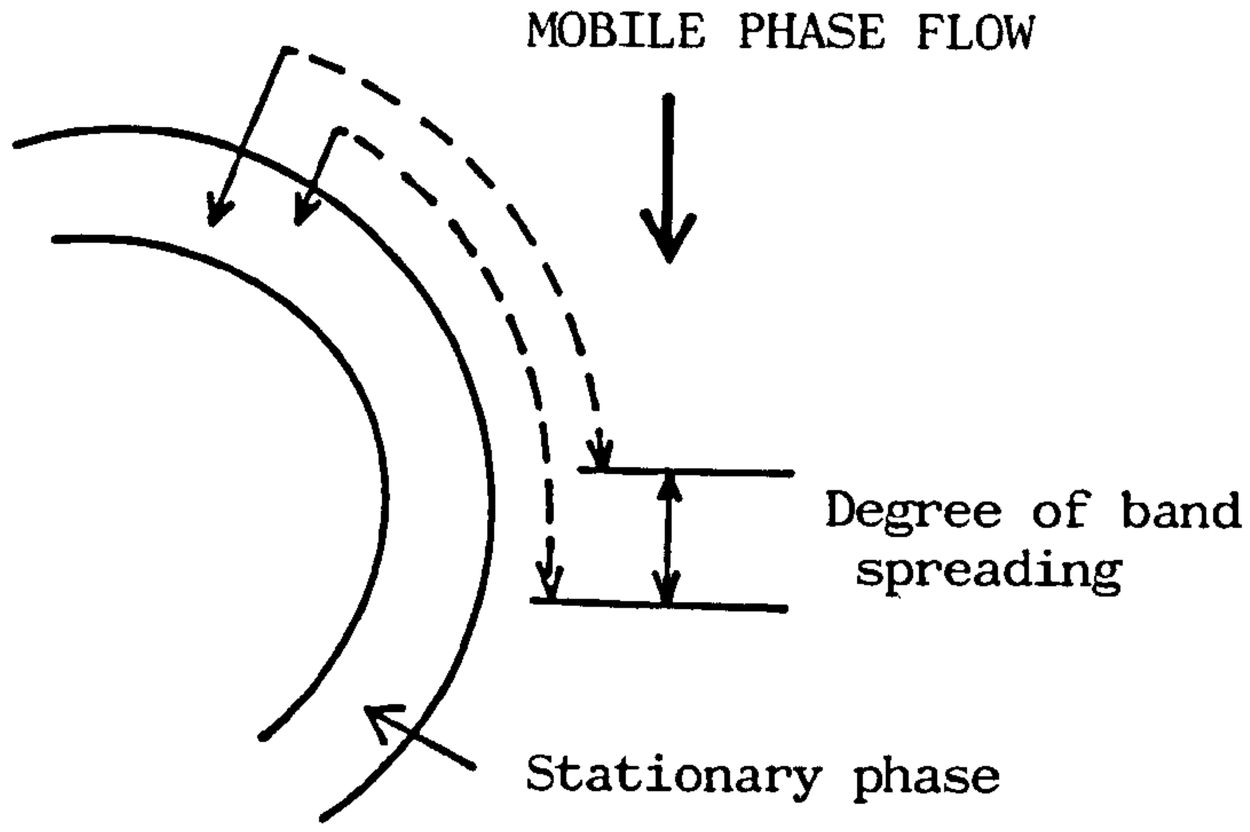


Figure 2.7: Stationary phase mass transfer[187]

In the mobile phase, solute molecules in the same flow path do not all have the same velocity, those close to a particle surface will move more slowly than those in mid-stream so causing an increase in band broadening, see figure 2.8.

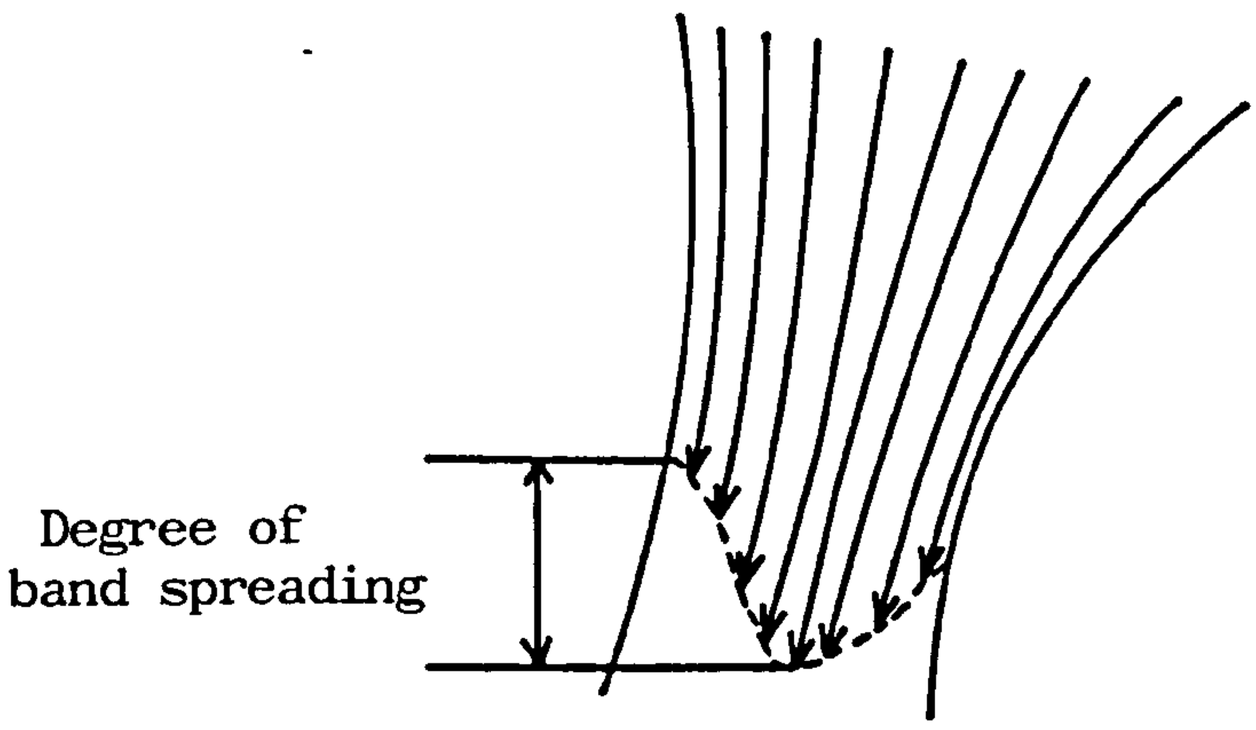


Figure 2.8: 'Moving' mobile phase mass transfer[187]

In LC, stagnant pockets of mobile phase trapped within the stationary phase exist. Solute molecules will diffuse through stagnant mobile phase to different degrees resulting in band broadening, see figure 2.9.

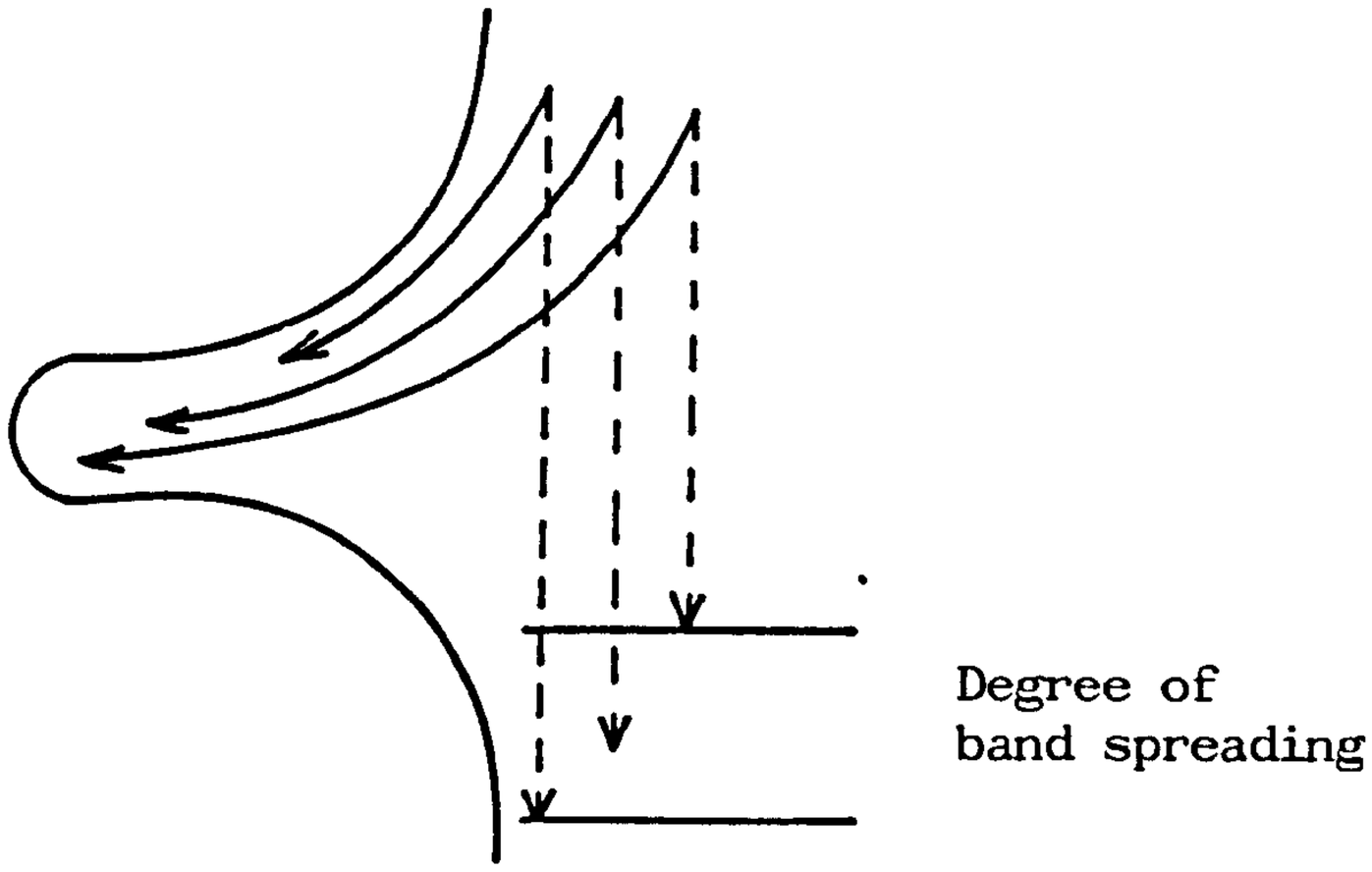


Figure 2.9: 'Stagnant' mobile phase mass transfer[187]

The contribution to the plate height due to the resistance to mass transfer in the mobile phase is proportional to

$$d_p^2 / D_M = C_{mobile}$$

In LC, radial mass transfer and eddy diffusion i.e. molecular diffusion coupled with uneven path lines, gives rise to a convective mixing or coupled term:

$$\frac{A}{1 + C_{mobile}/\bar{u}^{\frac{1}{2}}}$$

To minimize contributions to plate height due to the resistance to mass transfer in the mobile phase, a low viscosity mobile phase should be chosen so that diffusion of solutes is rapid. Also the mobile phase velocity and particle diameter should be kept to a minimum.

The undesirable effects of stagnant pools of mobile phase has led to the development of LC supports with solid cores or supports with very wide pores being chosen so that liquid flows in and out easily.

The overall plate height can be expressed as the sum of the individual contributions.

For GLC, the equation, known as the van Deemter equation, is stated below and shown graphically in figure 2.10.

$$H = A + B/\bar{u} + C_{stationary}\,\bar{u} + C_{mobile}\,\bar{u}$$

Eddy diffusion	Longitudinal or axial diffusion	Resistance to mass transfer in the stationary phase	Resistance to mass transfer in the mobile phase

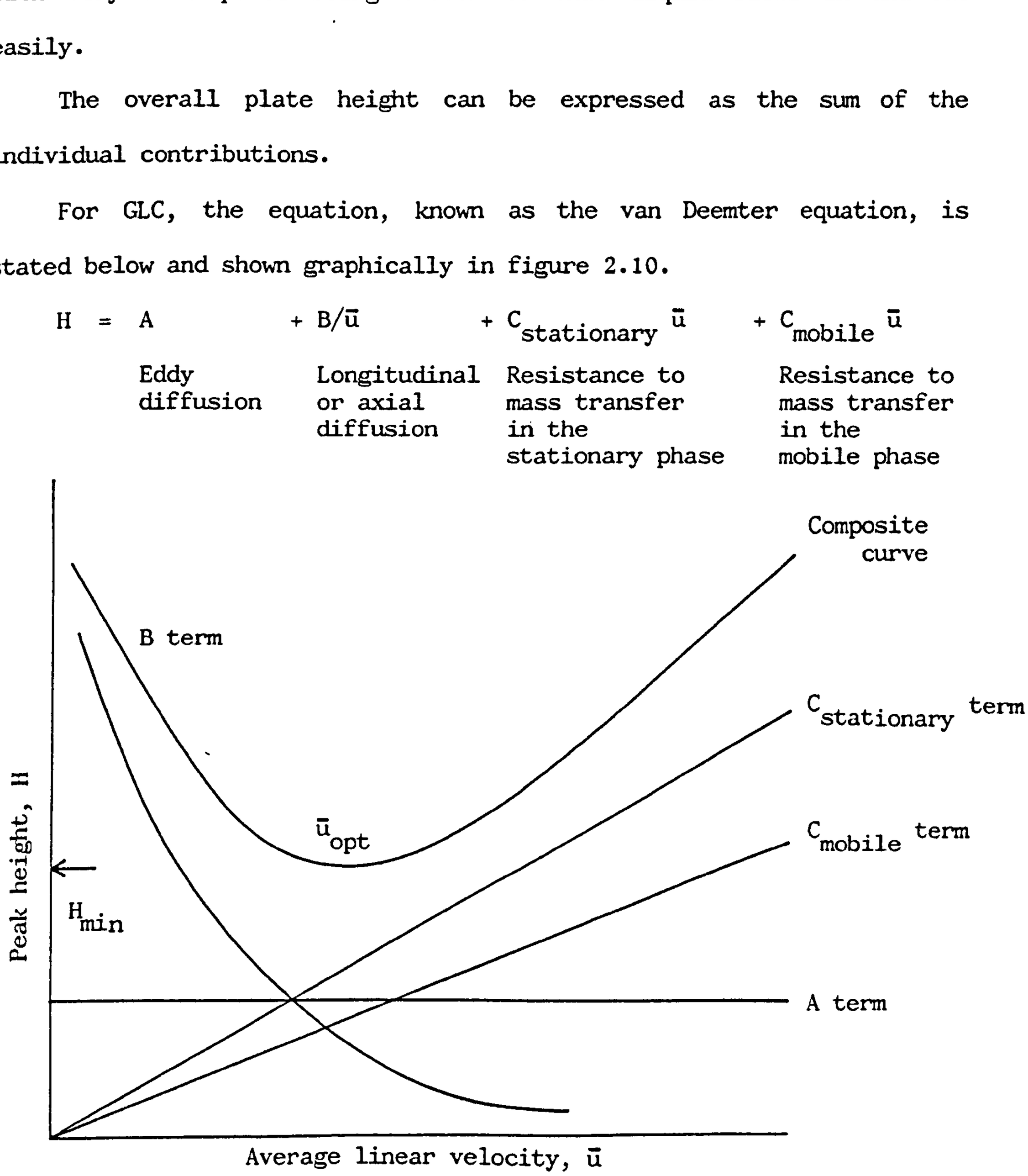


Figure 2.10: Typical $H/\bar{u}$ (van Deemter) curve for a gas chromatographic column

The plot of H vs. $\bar{u}$ shows a minimum value of H corresponding to an optimum average flow velocity for the best separation. Below this optimum velocity, H is dependent on the B term of the van Deemter equation i.e. longitudinal molecular diffusion is the controlling process. At high flow rates the mass transfer terms are the controlling features.

For LC, the equation takes the form:

$$H = B/\bar{u} + \frac{A}{1+C_{mobile}/\bar{u}^{\frac{1}{2}}} + C_{mobile}\,\bar{u}^{\frac{1}{2}} + C_{stationary}\,\bar{u}$$

Longitudinal or axial diffusion	Convective mixing	Resistance to mass transfer in the mobile phase	Resistance to mass transfer in the stationary phase

The relationships are shown graphically in figure 2.11.

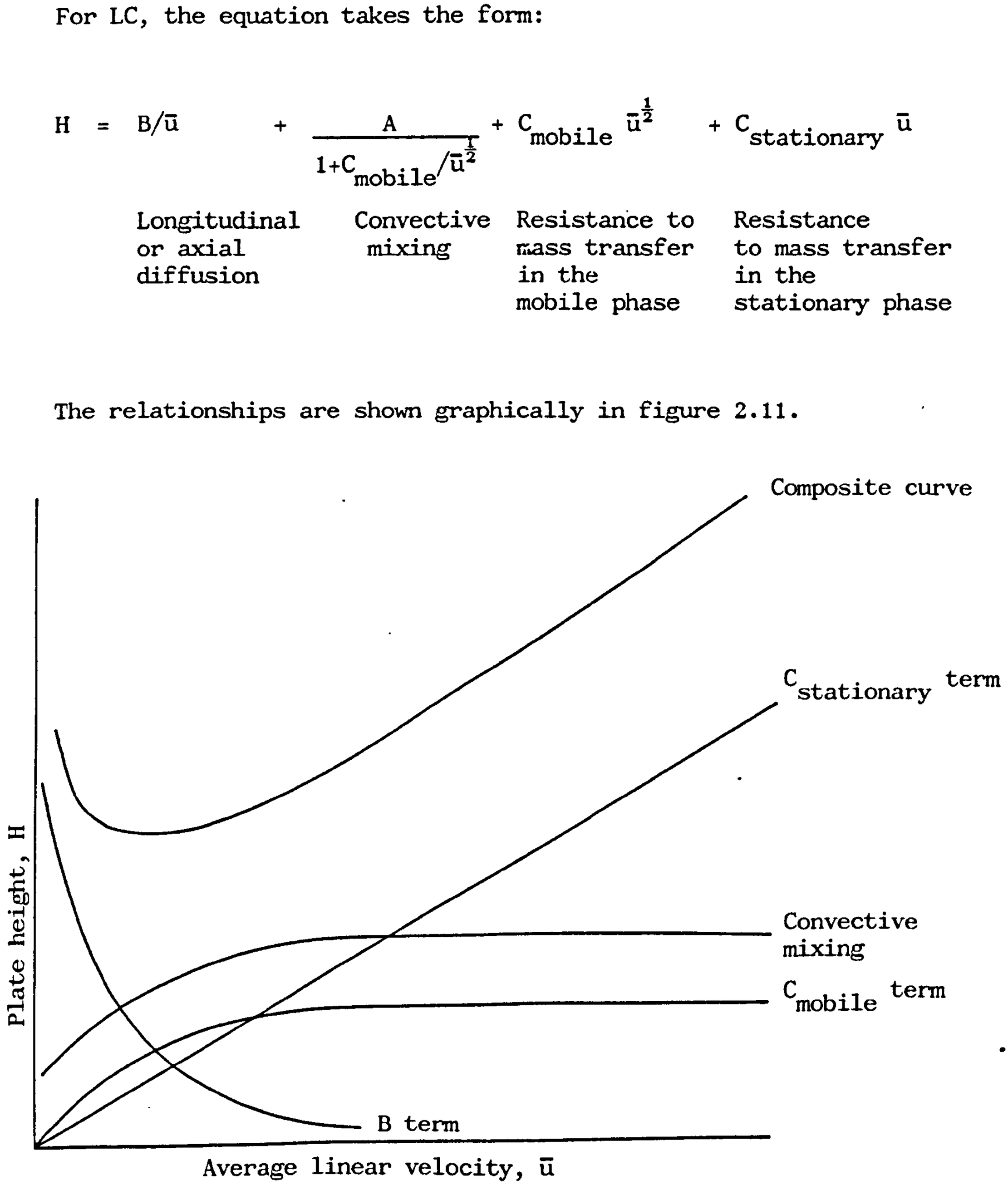


Figure 2.11: Typical $h/\bar{u}$ curve for a liquid chromatographic column

In LC where D_M is small, the B term is of little significance and hence the H vs. $\bar{u}$ curve rarely shows a minimum. The LC curve also shows a more gradual rise in H with increasing velocity than the corresponding GC curve. The flat slope of the H vs. $\bar{u}$ curve in LC means that high mobile phase velocities can be used without a serious loss in column efficiency. In all cases, as chromatograms must be obtained in a reasonable period of time, a compromise is made between column efficiency and flow velocity.

Taking into consideration all the above contributions to band broadening, to minimize H the mobile phase flow rate should be optimized, temperature increased, the solvent viscosity reduced and a more uniformly packed column with small diameter particles used.

Band spreading can also be caused by extra-column factors such as sample volume, connecting tubing and detector volume. Band spreading is proportional to the square of the sample volume, the square of the detector volume and also the fourth power of the diameter of the connecting tubing. Therefore sample volume and detector volume should be as small as possible and small diameter connecting tubing is essential.

For an optimum separation all aspects of the chromatography system must be given careful consideration.

2.3.5 Resolution

The usual goal of HPLC is the adequate separation of a given sample mixture. The quantitative measure of the separation of two peaks is expressed by the resolution R_s,

$$R_s = \frac{(t_{R_2} - t_{R_1})}{\frac{1}{2}(w_{b_1} + w_{b_2})} = \frac{2\,\Delta t}{w_{b_1} + w_{b_2}}$$

that is, the resolution R_s, of two adjacent bands, 1 and 2, is defined as being the distance between the two band centres, divided by the average band width. R_s can be improved by increasing the peak separation and by decreasing peak width. A resolution of about 1.5 is necessary to achieve baseline separation of two peaks.

2.3.6 Relative Retention

The ability of a particular stationary phase (or solvent) to produce a separation is expressed by the relative retention, α,

$$\alpha = \frac{t'_{R_2}}{t'_{R_1}} = \frac{t_{R_2} - t_M}{t_{R_1} - t_M} = \frac{k'_2}{k'_1}$$

The degree of separation of two peaks depends on α and the sharpness of the peaks (i.e. the number of theoretical plates possessed by the column). Two peaks with a large α value can be separated by a relatively inefficient column whereas, two components with a small value of α will require a highly efficient column, with a large number of plates, for separation.

2.3.7 Controlling Resolution

The resolution, R_s, can also be related to the capacity factor, k', the relative retention, α, and the number of theoretical plates, N, by the following equation

$$R_s = \frac{1}{4}\left[\frac{\alpha - 1}{\alpha}\right]\left[\frac{k'}{1 + k'}\right] N^{\frac{1}{2}}$$

The relative retention or selectivity, α, is governed by the interaction between the solute and the two phases. For a chosen stationary phase, the relative retention can be varied by changing the mobile phase composition. Temperature can also affect α. Methods for changing α are difficult to predict as they may involve changes in both mobile and stationary phases and may only result in a re-shuffling of the peaks with no real improvement in resolution.

The capacity factor, k', can have a dramatic effect on the resolution. If k' = 0 then there will be no separation. As k' values increase, there is a marked increase in R_s; the optimum range for k' is $1 \leqslant k' \leqslant 10$ and can be controlled by the composition of the mobile phase. If k' is increased further, peak heights decrease rapidly and hence the peaks become more diffuse and difficult to detect and the analysis time increases.

If k' is within the optimum range and resolution is still poor, an increase in N or the column efficiency can improve R_s

$$R_s \propto (L/H)^{\frac{1}{2}}$$

Therefore an increase in column length will result in improved resolution, however R_s only increases as the square root of the column length. Increased column length also means an increase in analysis

time or an increase in the column inlet pressure if the retention times are to remain constant. An alternative approach is to increase N by lowering the mobile phase flow rate, however this again causes longer analysis times and only modest increases in resolution.

Temperature has only a small effect on retention and resolution in HPLC as ΔH is much smaller in LC than in GC.

$$\frac{d \ln k'}{dT} = \frac{\Delta H}{RT^2}$$

Temperature control is used to keep k' values constant. Increased temperature reduces the viscosity of the mobile phase and also increases the solubility of sparing soluble substances in the mobile phase, so reducing analysis times.

2.4 Optimization of Column Performance

k' values are controlled by solvent strength. A 'weak solvent' may be used to increase k' values and to reduce k' a 'stronger solvent' may be used. The mobile phase usually consists of a mixture of solvents which can be altered to obtain k' values in the optimum range, for the component(s) of interest. In a sample containing components of interest with widely differing k' values the early peaks may not be resolved adequately and later peaks may be very broad and difficult to detect. The analysis time may also be unnecessarily long. No single isocratic solvent composition can elute all substances with good resolution in a reasonable time. The k' values of the different components may be optimized during the separation by changing the

composition of the mobile phase. Weakly adsorbed components (early peaks) may be eluted using a weak solvent and a change to stronger solvent mixtures would also allow strongly adsorbed components (later peaks) to elute with k' values in the optimum range. The change in mobile phase composition is normally carried out continuously and is known as gradient elution. Commercially available gradient programmers are used to control the composition of the mobile phase.

2.4.1 Gradient Elution

Selection of a particular gradient can only be made by trial and error. There are three important considerations:

(a) the choice of solvents A and B,

(b) the gradient shape, and

(c) the gradient steepness.

The choice of the initial and final solvent compositions is important in obtaining the desired separation with adequate resolution in a reasonable time. If the initial solvent, solvent A, is too weak valuable time will be lost, resolution is not improved and sensitivity is decreased, however if solvent A is too strong, the components of interest will elute quickly without adequate resolution. All peaks of interest should be eluted during the gradient or soon after the final composition has been reached. If this is not the case then solvent B is too weak and the final components of interest may have increased band widths or remain on the column. Ideally solvent A must be weak enough to give a good separation of the components with low k' values and solvent B must be strong enough to elute the components with large k' values in a reasonable time

with a k' value closer to the optimum.

Depending upon which commercial programmer is being used, different gradient shapes may be available, as shown below.

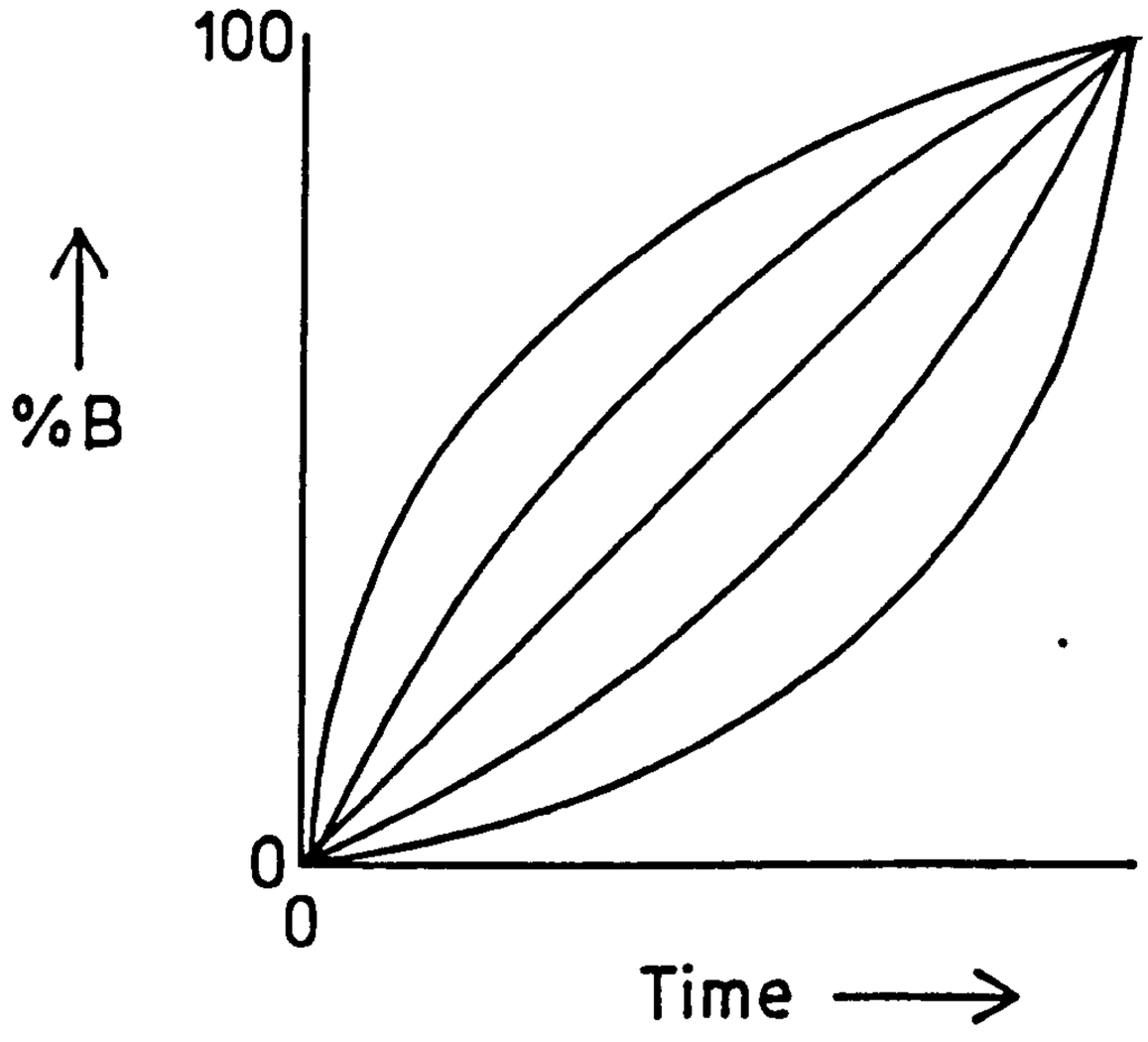


Figure 2.12: Gradient Shapes

A change in gradient shape will cause a change in the k' values of the components. In order to illustrate the effect of both convex and concave gradients on a particular chromatogram, let a linear gradient give an optimum separation with all peaks being resolved and of equal width, see fig. 2.13(b). A convex gradient, with an initial rapid increase in percentage of B in the mobile phase, leads to the elution of early peaks in the chromatogram with lower k' values and so they are sharper but less well resolved. For the peaks eluting later in the chromatogram there is an increase in resolution but also an increase in peak width as the rate of change of percentage of B decreases, see fig. 2.13(a).

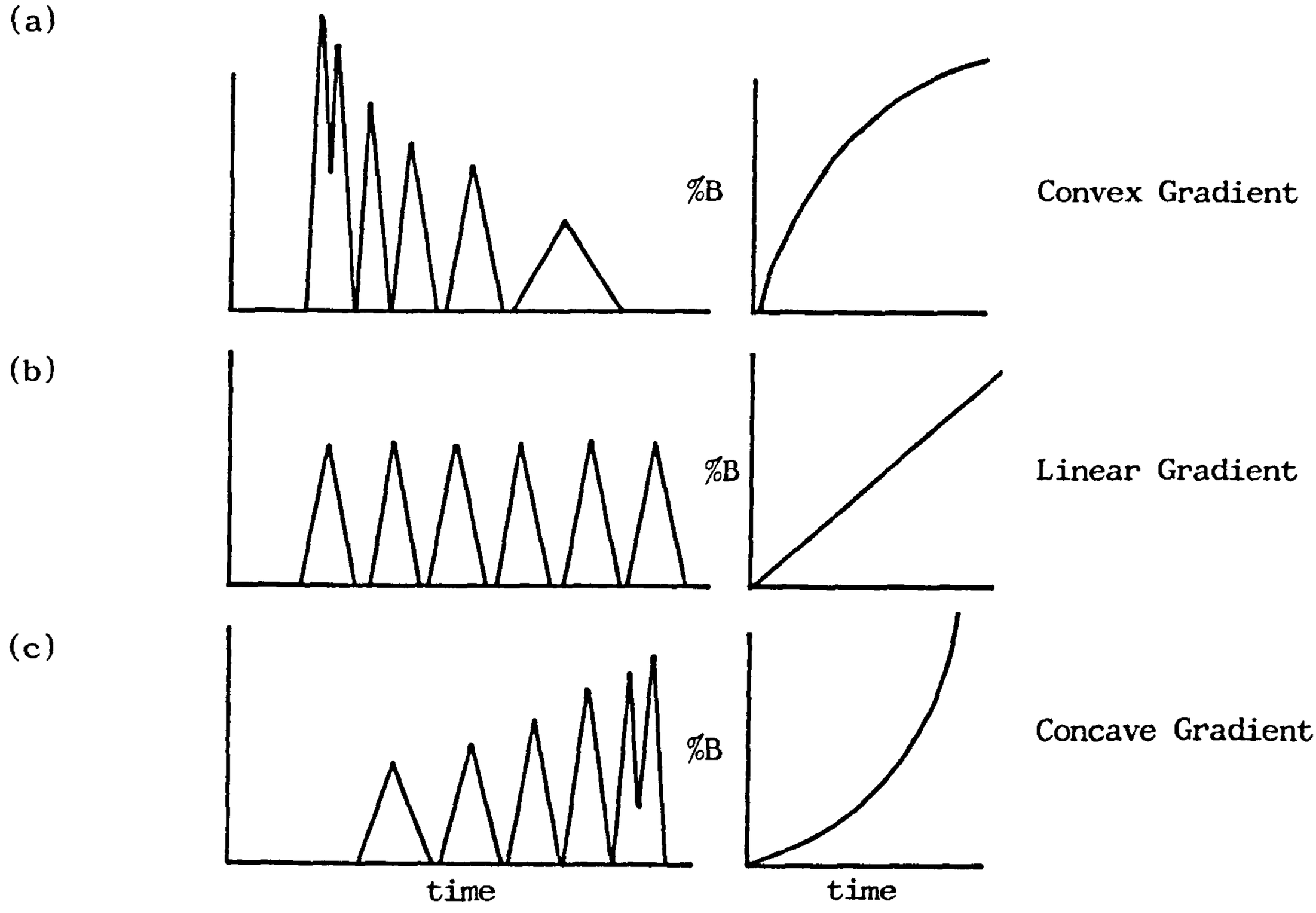


Figure 2.13: Effect of gradient shape on the resulting chromatogram [186]

A concave gradient has the opposite effects - wider peaks initially which are well resolved, but sharper, less resolved peaks towards the end of the chromatogram, see fig. 2.13(c).

In some cases, even the use of the weakest possible solvent A results in poor resolution of the initial components in the chromatogram. By using the so-called 'gradient delay', an improvement is sometimes possible. The gradient delay allows the initial components to elute under isocratic conditions. The gradient is then started after a time lag of t mins.

In an isocratic separation, a decrease in k' decreases resolution and increases sensitivity so in gradient elution resolution decreases and sensitivity increases with increasing gradient steepness.

Increasing gradient steepness has the same effect as increasing the solvent strength in isocratic elution, the steeper the gradient, the shorter the time of analysis.

The main advantage of gradient elution is that it gives maximum resolution per unit time. However, in some cases it may be responsible for a lack of reproducibility due to changes in the mobile phase composition leading to variation in retention times.

2.5 LC Instrumentation

Modern liquid chromatographs basically consist of a high pressure solvent delivery system to pump mobile phase through relatively short, narrow columns containing the stationary phase and a sensitive detector system. Figure 2.14 is a general schematic diagram of the equipment used for modern LC systems.

2.5.1 Solvent Delivery System

It is important to degas and filter all solvents before they pass into the HPLC system. Degassing is required to remove dissolved gases (e.g. oxygen) and is carried out by heating under reflux, evacuation coupled with ultrasonic vibration or the use of a helium sparge. Solvents which are not degassed tend to produce (air) bubbles towards the column outlet and these interfere with the functioning of the detector. Solvents which are most prone to dissolved gases are water and alcohols in which air is quite soluble.

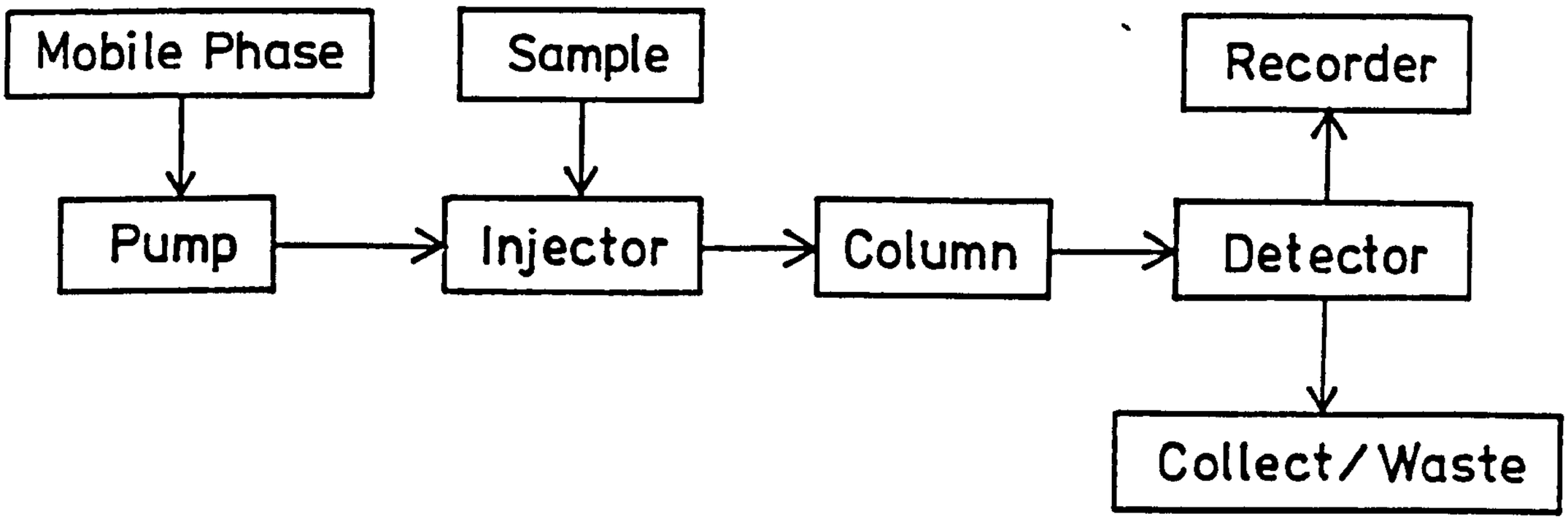


Figure 2.14: Schematic diagram of a modern HPLC system

Filtration using a fine mesh filter less than 0.45 μ is also desirable, to remove any small particles which can impair the functioning of the (non-return) valves in the pump.

The pump is a very important component of the modern LC system. Pumps may be classified into two main types: (i) constant flow pumps and (ii) constant pressure pumps. Constant flow/volume pumps are considered better than those generating constant pressure.

The most widely used pumps are reciprocating pumps of which there are several different types including single-head and dual-head. These pumps have small volume chambers with reciprocating pistons. Each piston stroke involves solvent being drawn in from the solvent

reservoir through a non-return valve and then being pushed out through another valve to the column. Although these pumps usually give a constant flow rate, regardless of column back pressure or mobile phase viscosity, they produce a pulsating flow which can give rise to base-line noise. The use of dual or triple pump heads or other damping devices can smooth the flow of solvent delivery to the column. Reciprocating pumps are suitable for use with gradient elution and because of their small internal volume solvent changes are rapid and accurate.

2.5.2 Pump/Equipment for Gradient Elution

There are two types of gradient system depending on whether the mixing of the solvents takes place at low or high pressure.

High pressure gradient programmers require two high pressure pumps and a gradient programmer, see figure 2.15 . The two solvents are pumped at high pressure into a mixing chamber before being pushed from the mixing chamber to the top of the column. Many different gradients are possible as the output from each pump can be controlled by the programmer. However, reciprocating pumps operate with poor precision at low flow rates (< 0.1 ml min^{-1}). At the beginning and end of a gradient run small amounts of one or other of the solvents may be required so this is a limiting factor.

There is a trend towards the use of low pressure gradient programmers now. In low pressure gradient systems the solvents are mixed at atmospheric pressure and then pumped by a single high pressure pump to the column, see figure 2.16. Low pressure gradient systems

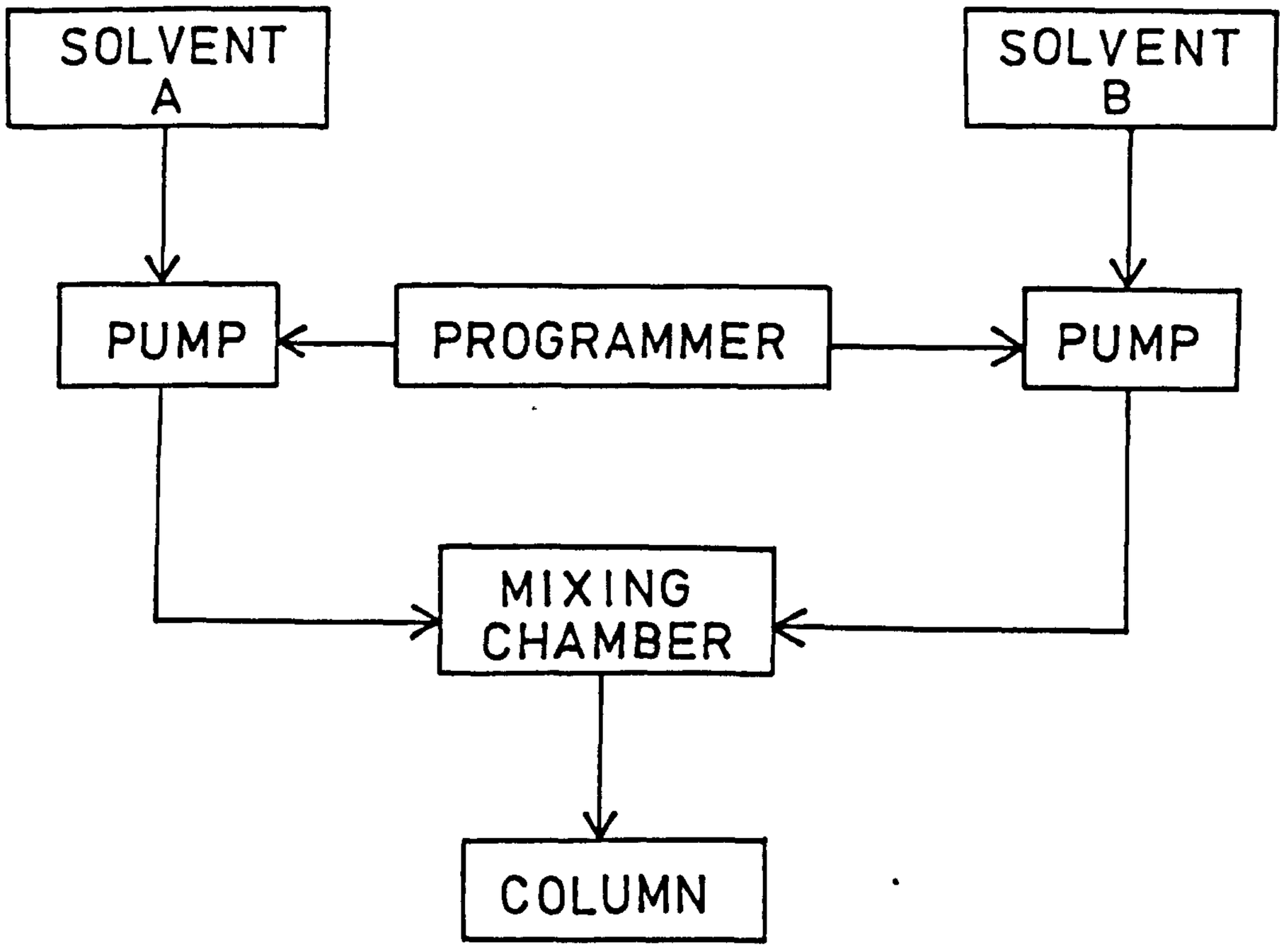


Figure 2.15: High-pressure mixing gradient system

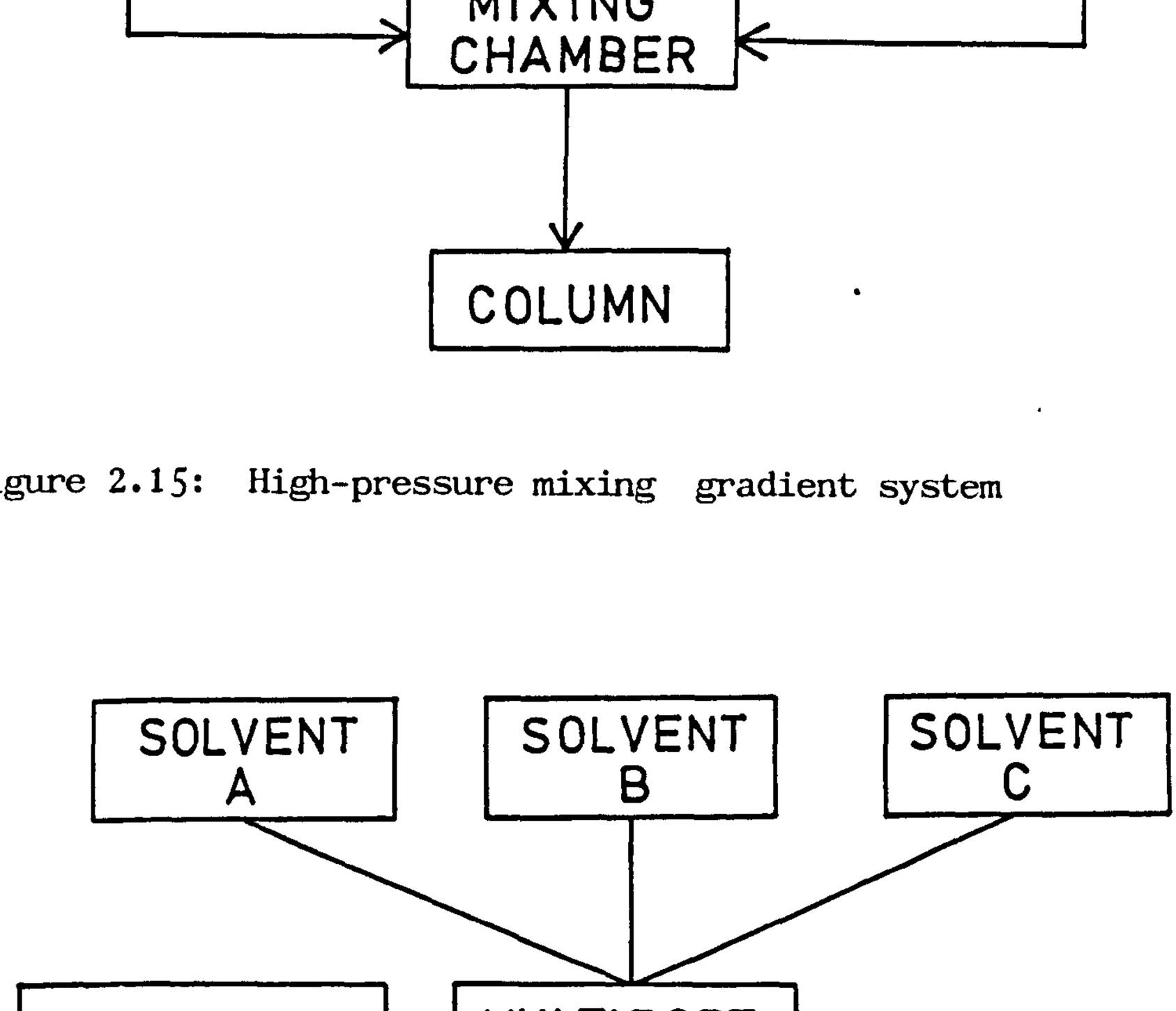


Figure 2.16: Low-pressure mixing gradient system

have several advantages over high pressure systems. They require only one pump therefore they are less expensive. They are capable of mixing several different solvents whereas two solvents are normally used in high pressure systems. Solvent volume changes which are produced as a result of mixing occur at low pressure whereas these volume changes may cause a change in the flow rate in the high pressure system. Output in the low pressure system depends on the precision of only one pump. The overall effectiveness of the gradient system depends on the efficient mixing of the liquids. Use of a precise valving system for low pressure work ensures delivery of thoroughly mixed and degassed solvents to the pump resulting in reproducible gradient separations.

2.5.3 Sample Introduction

Ideally, the sample should be introduced into the pressurized column as a narrow plug onto the top of the column, to minimize peak broadening. Sample injection must also be reproducible and convenient to use. There are two methods of sample introduction, syringe injection, and sampling valves and loops.

In the syringe-septum method, the sample is injected with a high pressure syringe through a self-sealing elastomeric septum. At high pressures maintaining a leakproof septum and also inserting the needle into a pressurized system is difficult so reproducibility is rarely better than 2%. Another method used is the 'stopped flow' technique where the mobile phase flow is switched off or diverted while the injection is made. This method can be used at very high pressures, however it can lead to retention time uncertainty.

The most widely used method of sample introduction is the injector valve or loop. The small loop (10-500 µl) is initially filled with sample using a syringe. By valve switching the sample in the loop is introduced onto the top of the column as the flow of mobile phase is diverted through the loop before reaching the column. Although increased band spreading may result and the technique is wasteful with sample, loop injections are reproducible with valve error < 0.2% and the technique is amenable to automation. In addition to external loops (10-500 µl) which are interchangeable, very small fixed volume internal loops (1-3 µl) are also available. Loop-valve injections are now used almost to the exclusion of syringe injections.

2.5.4 The Column

The column, the core of the chromatograph, is usually constructed of heavy wall glass or stainless steel tubing so as to withstand the high pressures. Glass columns are superior to those made from steel. Performance is improved if the stainless steel is polished to give a smooth internal surface. This reduces the mobile phase channelling near the wall/packing material interface. The internal diameter of the column is precision bored.

Flexible walled cartridges (10 cm in length with an 8 mm bore), which are compressed radially in a compression module, are also now available (from Waters), see figure 2.17. The efficiency of a rigid-walled column is less than that which is attainable when flexible column walls are used. With rigid-walled packed columns the dispersion of the mobile phase near the column wall is greater than that in the centre of the column. This is known as the 'wall effect'. Taking

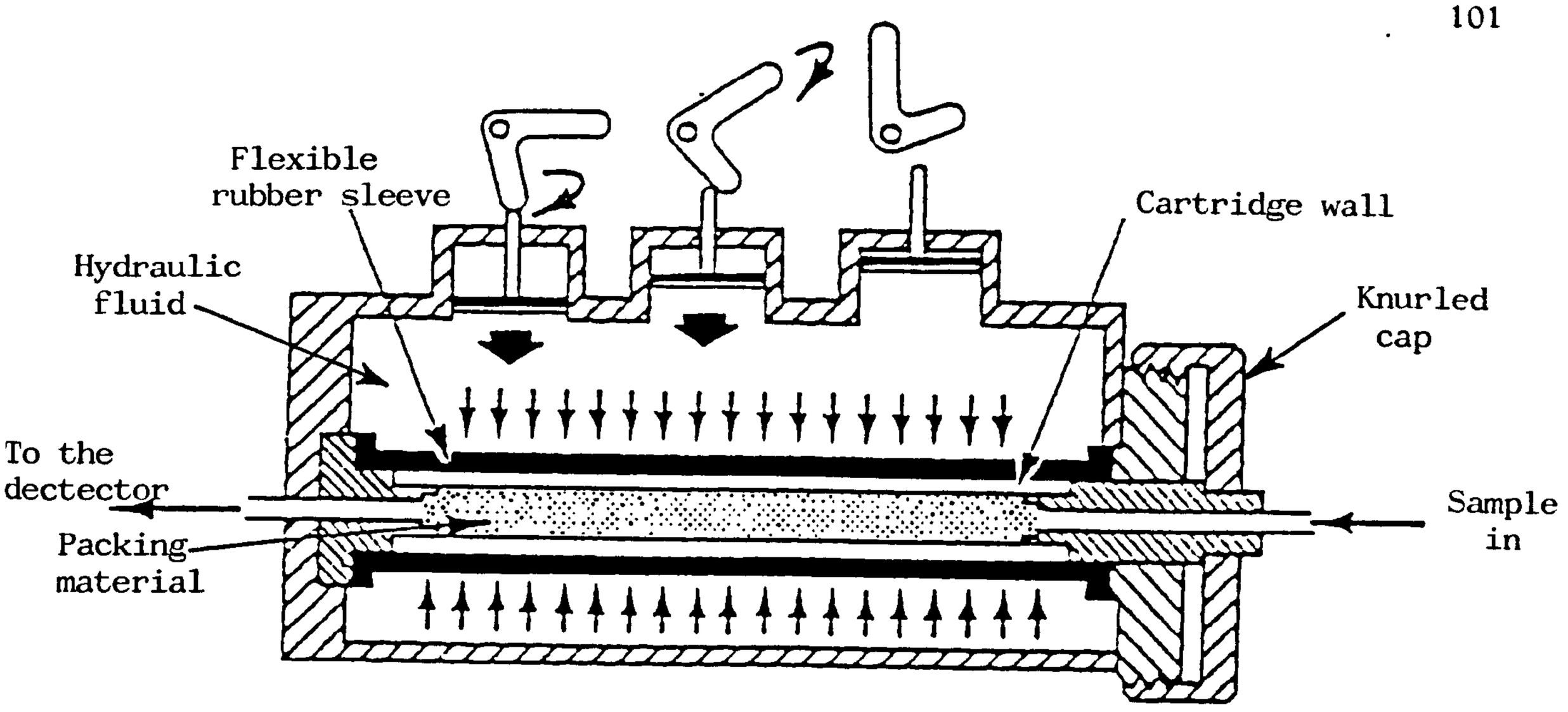


Figure 2.17: Waters Radial Compression Module (cross section diagram)[188]

a perpendicular cross-sectional slice through the column, at different positions in the flow stream, there are different velocities caused by the different local permeabilities through these channels which can arise due to wall irregularities and poor packing. The movement of the solute band through the column is therefore not uniform in conventional packing structures and is a source of band broadening, see figure 2.18. Using a flexible walled column hydraulic pressure is applied along the radial axis via a fluid, such as glycerol, contained within a plastic sleeve. The column wall is able to mould around the column packing, see figure 2.19. This gives a more homogeneous packed bed. Wall effects, column channelling and voiding can be virtually eliminated and this increases column efficiency.

Columns with an internal diameter of 5 mm provide a good compromise between sample capacity, the amount of packing used, solvent required and column efficiency. A 2 mm bore packed column requires a much higher

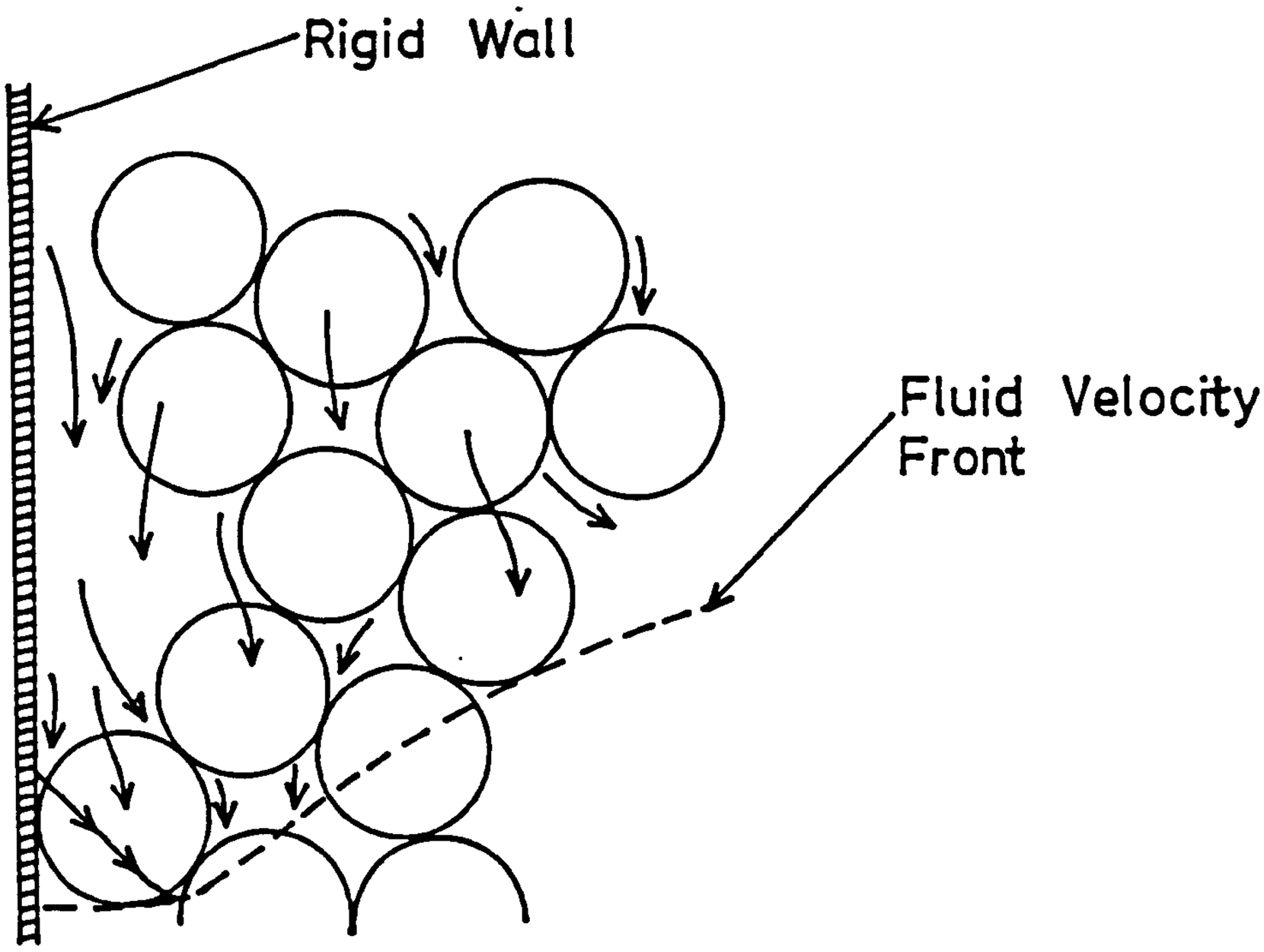


Figure 2.18: Flow through a standard stainless steel column

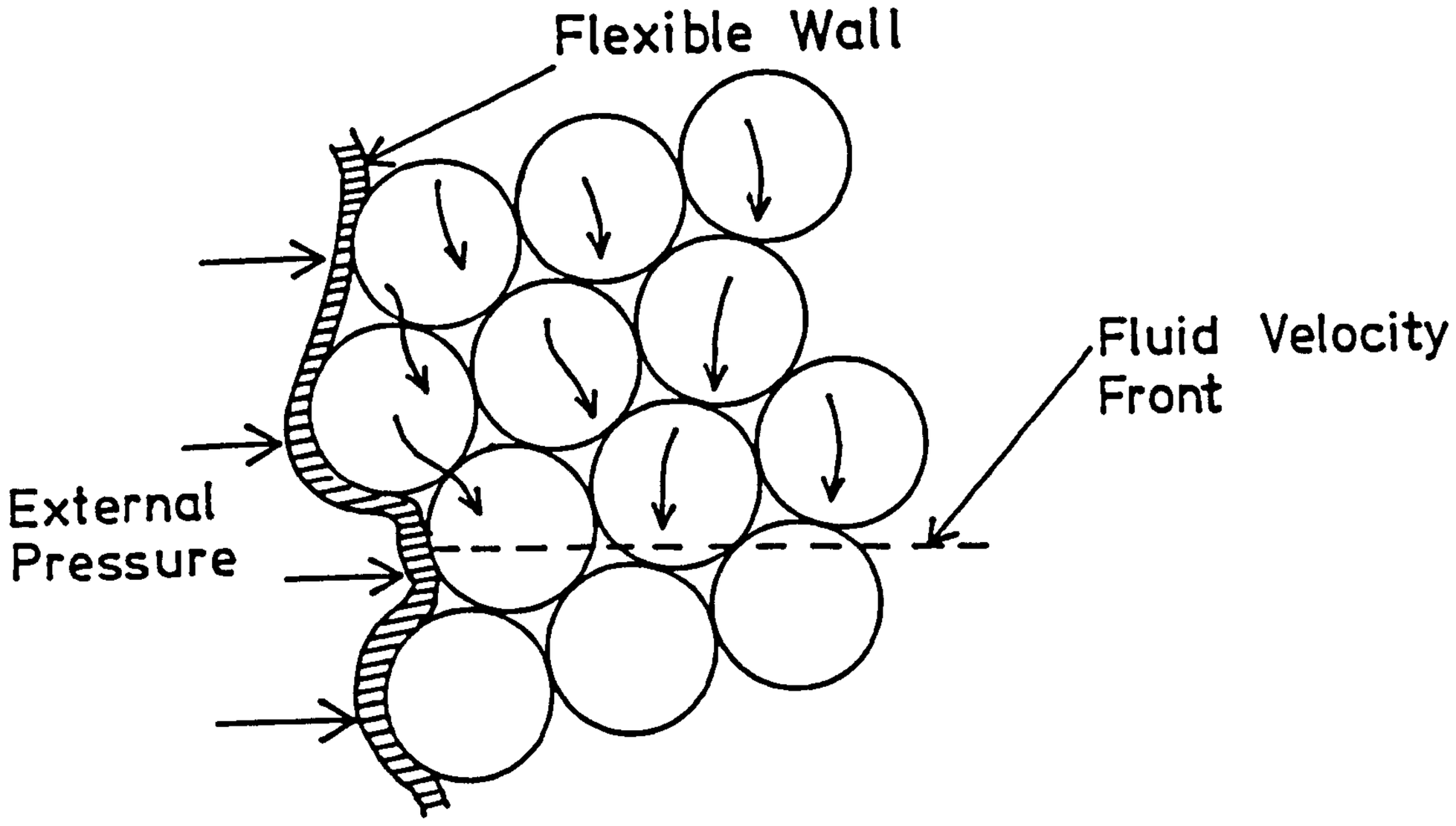


Figure 2.19: Flow through a radially compressed cartridge

inlet pressure as the same length of 5 mm column for the same flow rate. Large bore columns require considerably more packing material for the same length. Extra-column band broadening due to fittings, tubing, detector and injector dead volume becomes less important with increasing column internal diameter as a larger bore column gives broader peaks.

Guard columns, short protection columns < 5 cm in length, may precede the analytical/separation column to filter out unwanted material. Use of such a column can increase the lifetime of the analytical (separation) column. Both analytical and guard columns should be packed with similar packing material. Guard columns can lead to a slight decrease in efficiency but usually this is not critical.

2.5.5 Detectors

In liquid chromatography no single detector exists which satisfies all the necessary requirements for an ideal detector. The detector must be selected for a particular problem and so several detectors are available. The most widely used detectors include:

(i) the UV/visible detector

(ii) the refractive index (RI) detector

(iii) the fluorescence detector

(iv) the electrochemical detector

(v) the infrared (IR) detector and

(vi) the mass spectrometer.

(v) and (vi) are used, but less frequently.[194]

The detectors are of two basic types. The bulk property detector, e.g. RI detector, involves the differential measurement of a physical property of the mobile phase with and without the eluting solute. The solute property detector, e.g. the UV detector and the electrochemical detector, involves measurement of a physical property characteristic of the solute which is not exhibited by the mobile phase. Some solute property detectors require the removal of the mobile phase before detection, e.g. the mass spectrometer. Bulk property detectors tend to be relatively insensitive (microgram range), although they are universal, responding to practically every solute. Solute property detectors only respond to a limited range of solutes but sensitivity is high (nanogram levels), and temperature control is not essential.

UV detectors are the most commonly used of all the detectors, although they are restricted to solutes which absorb UV radiation. The original detectors were single or dual wavelength instruments (254 nm and/or 280 nm). Variable wavelength detectors are now available covering the range 210-800 nm and these offer several advantages over fixed wavelength instruments, such as increased sensitivity, since the wavelength of maximum absorption of the component of interest can be chosen, and greater selectivity since a wavelength can be chosen where the solutes of interest absorb while others do not. The mobile phase should not absorb in the UV region or absorb only weakly. The UV cutoff valve is considered to be the wavelength below which the solvent will absorb more than 1.0 absorbance unit in a 1 cm path cell, e.g. the UV cutoff for methanol is 210 nm.

Very pure solvents must also be available as the detection limit

is dependent on solvent purity. The detector is a solute property detector and so sensitivity is high, it has low noise characteristics, is relatively insensitive to temperature changes and fluctuations in flow rate and ideally suited to gradient elution work. Many of the components of interest in LC are UV active.

2.5.6 Quantitation/Data Handling

The output from the detector is usually recorded by a chart recorder and if an integrator or computerised data handling system is used peak retention times and peak area measurements may also be recorded. Some systems allow for immediate component identification and their concentrations in the sample.

2.6 The Separation Problem

Success in HPLC analysis is the separation of a mixture into its individual components. This separation is dependent upon the choice of the chromatographic mode and a packing material and eluent compatible with this type of separation. Once achieved, the separation will require optimization to give the desired resolution.

The nature of the mixture of components of analytical interest and ultimately the nature of the sample matrix in which they are present determines how critical the choice of column packing is. The analysis of a compound and its associated metabolites, as in the case of nicotine and its structurally related breakdown products, is a challenging problem in terms of column selection, requiring careful attention. It must also be remembered that the separation achieved will as an end result be applied to urine samples where sensitivity and selectivity will be of critical importance.

2.7 Choice of Detector

UV was the chosen method of detection as nicotine and the metabolites, cotinine and nicotine-1'-N-oxide, all absorb UV radiation. Most of the HPLC studies carried out previously have also used UV detection,[166,168-171,179,181] even though UV seemed to lack the required sensitivity and selectivity for measuring low levels of these substances in both urine and plasma. Mousa et al.[172] used electrochemical detection for the determination of nicotine and N-methylnicotinium ion, a minor metabolite. However, cotinine, 3' hydroxycotinine and

nicotine-1'-N-oxide were not observed in the sensitivity range employed for the analysis. Electrochemical detection proved to be a very sensitive and selective method though not applicable to many metabolites. Liquid chromatography-mass spectrometry (LC-MS) is also now commercially available. LC-MS has not been reported in the literature up to the time of writing, however there are many examples of MS being used as the detector in GC analysis.[148,158-161,184] Mass spectrometry is a very sensitive method of detection, however in most cases its initial capital cost is prohibitive and, as it is a complex instrument, it is unsuitable and an unlikely choice for routine analysis.

The differential refractometer detector is also used widely in LC. This detector monitors the difference in refractive index between the pure mobile phase and the mobile phase plus sample as it elutes from the column. It has not been used for the detection of nicotine and its metabolites due to inferior sensitivity and stability as compared to UV detection. Refractive index (RI) detectors are sensitive to changes in the mobile phase composition and so they are not suitable for gradient elution work.

2.7.1 Selection of the detection wavelength

Some of the preliminary studies were carried out using a fixed wavelength UV detector, λ = 254 nm, although a variable wavelength UV spectrometer was available for all quantitative work. A variable wavelength detector is desirable as it enables the wavelength to be adjusted for particular sample components so providing optimum sensitivity and selectivity.

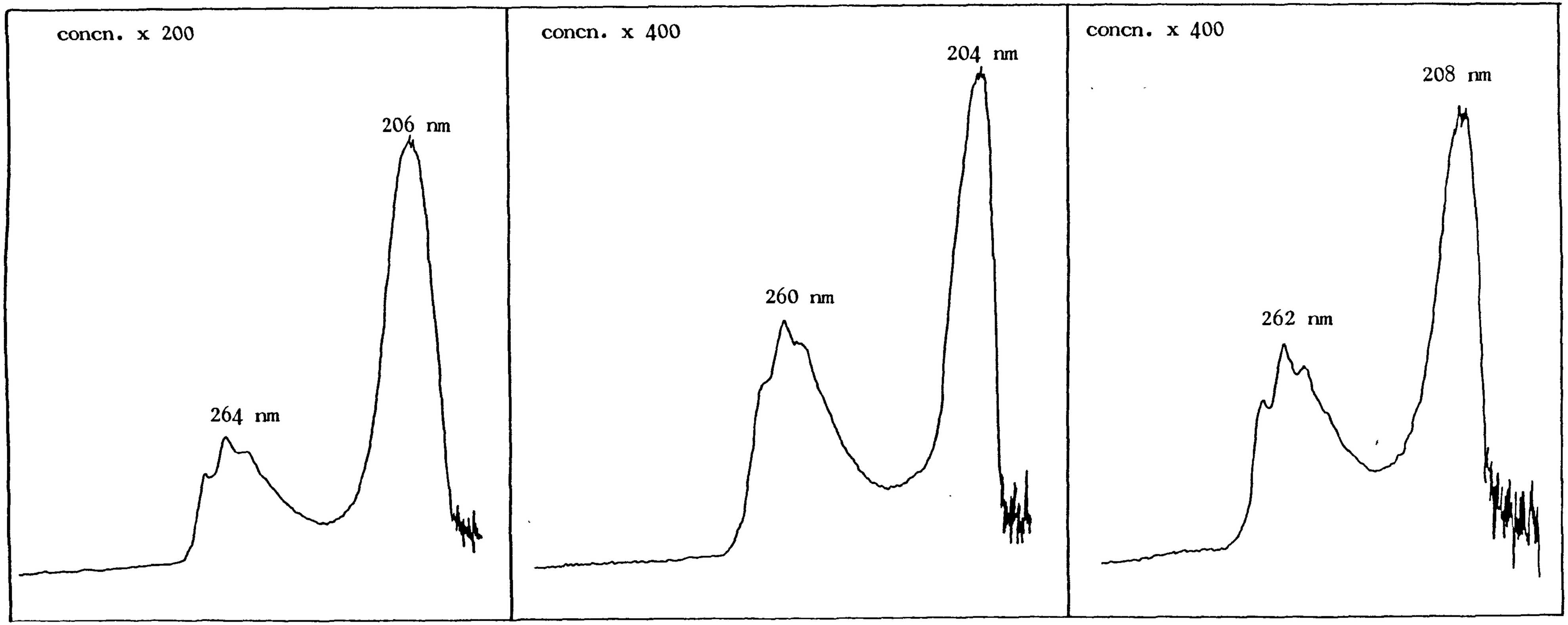
The UV spectra of the standards of interest were recorded and are shown in figures 2.20. A detection wavelength of 260 nm was chosen.

2.8 Ion Chromatography

Cundy and Crooks[171] applied cation exchange chromatography to the analysis of nicotine, three oxidative metabolites, nicotine-1'-N-oxide, cotinine and nornicotine, and four potential methylated metabolites. A separation was achieved using a Partisil 10 SCX column with an eluent of 0.3M sodium acetate-methanol (70:30) buffer, pH 4.5 initially, followed by a gradient of triethylamine rising from 0 to 1.0% over a 10 minute period, to elute the more strongly bound standards. In a urine sample from a guinea pig, Cundy and Crooks detected cotinine and nicotine-1'-N-oxide as major urinary metabolites, small amounts of nornicotine and unmetabolized nicotine. The guinea pig produced only one identifiable methylation product, the N-methyl-nicotinium ion.

The work of Cundy and Crooks[171] was taken as a starting point and cation-exchange chromatography was used in an attempt to separate the protonated alkaloids at low pH. A Partisil 10 SCX column (30 cm x 2 mm ID) was used (see table 2.2), with a methanol (70%), sodium acetate (30%) (0.3M, pH 4.5) mobile phase. Nicotine, cotinine and nicotine-1'-N-oxide were eluted from the column virtually unretained and not separated from one another. A separation of the three standards had been achieved by Cundy and Crooks although cotinine had not been

Figure 2.20: UV Spectra
Standards (10 μg ml^{-1}) at pH 7 in H_2O were used.



Cotinine

Nicotine

Nicotine-1'-N-oxide

TABLE 2.2: Specifications of the Stationary Phases evaluated for the analysis of Nicotine and its Metabolites by ion-exchange chromatography

Packing Material	Manufacturer	Length (cm)	I.D. (mm)	dp (μm)	Functional Group	Further Details
Partisil SCX	Whatman	30	2	10	Sulphonic acid	Cation exchange

TABLE 2.3: Specifications of the stationary phases evaluated for the analysis of nicotine and its metabolites by reverse phase ion-interaction chromatography

Stationary Phase	Manufacturer	Length (cm)	ID (mm)	dp (μm)	Particle Shape *	Carbon Loading (%)	Mean Pore Diameter (nm)	Surface Area $m^2 g^{-1}$	Surface+ Coverage $g m^{-2}$	Degree of End Capping
Hypersil ODS	Shandon-Southern Products	10	5	5	S	10	9-10	200	0.50	Monolayer, fully end-capped
Spherisorb ODS 1	Phase Separations Group	10	5	5	S	7	8	220	0.32	Partially end-capped
μ-Bondapak ODS	Waters Associates	30	3.9	10	I	10	12.5	300-500	0.33	End-capped

* I = Irregular
S = Spherical

+ surface coverage ($g m^{-2}$) = $\frac{\text{C loading (\%)} \times 1000}{\text{surface area } (m^2 g^{-1}) \times 100}$

completely resolved from the compounds eluting in the void volume, those which elute with little or no retention. Variation of the available parameters, such as the percentage of methanol, the molarity of sodium acetate in the mobile phase and the pH of the buffer, in an attempt to achieve a separation of the standards, proved to have little effect. This was in agreement with the findings of Cundy and Crooks.[171] Hence, cation exchange chromatography was abandoned as this mode of chromatography showed little flexibility.

2.9 Reverse Phase Chromatography

In the reverse phase mode the stationary phase is less polar than the mobile phase. Many interfering species present in biological systems are ionic in nature, which results in possible interfering ions showing little affinity for the column and eluting after the void volume without being retained. Most importantly, the reverse phase mode is very versatile with additional operational variables not available in the normal phase or ion-exchange modes and therefore it was hoped that this added flexibility would help effect a separation of nicotine and its metabolites.

2.9.1 Reverse Phase Ion-Interaction Chromatography

Cundy and Crooks[171] also reported an attempt to develop an analytical method for the quantitation of nicotine and its methylated metabolites using reversed phase columns with an ion-interaction agent at a low pH (RP-IIC). However, it was abandoned due to the

very high affinity of the methylated nicotine paired ions for the reversed phase packing material. As this study was not directly concerned with the methylated metabolites, RP-IIC was investigated as a possible method for the analysis of nicotine, cotinine and nicotine-1'-N-oxide.

The mobile phase included the ion-interaction agent, pentadecyl-fluoro-octanoic acid (PDFOA). Various types of reversed phase (ODS) column materials were evaluated to find the most suitable. These columns are listed in table 2.3. Initially the mobile phase consisted of 0.3M sodium acetate, pH 4.5 : 100 $\mu g\ ml^{-1}$ PDFOA in MeOH (70:30). The available parameters were altered in an attempt to effect a separation. Ratios of buffer to methanol other than 70:30 were examined, the concentration of PDFOA was varied from 100-1000 $\mu g\ ml^{-1}$ and, pH being an important factor influencing retention in RP-IIC, the pH of the acetate buffer was controlled using CH_3COOH. Triethyl-amine (Et_3N) and diethylamine (Et_2NH) were both used as anti-tailing agents, being added as 0.1% of the mobile phase.

Using the Hypersil ODS column (10 cm x 5 mm ID) and a mobile phase which initially consisted of CH_3COONa 0.3M, pH 4.5 : 200 $\mu g\ ml^{-1}$ PDFOA in MeOH (70:30) and 0.1% Et_3N, nicotine, cotinine and nicotine-1'-N-oxide were chromatographed. Nicotine-1'-N-oxide and nicotine were poorly retained and were not completely resolved from the solvent front. On changing the concentration of PDFOA in MeOH from 200 $\mu g\ ml^{-1}$ to 1000 $\mu g\ ml^{-1}$, all three were again chromatographed, as shown in figure 2.21. Slight variations in the retention times were noted, see table 2.4, and in order to ensure an excess of the IIA, 1000 $\mu g\ ml^{-1}$ PDFOA was chosen for all future RP-IIC work.

Figure 2.21: Reverse Phase Ion-Interaction Chromatography of Individual Standards on a Hypersil ODS Column with different concentrations of Ion-Interaction Agent in the Mobile Phase

Parameters:

Column: Hypersil ODS (10 cm x 5 mm ID)

Detection: UV at 254 nm

Flow Rate: 1 ml min^{-1}

Sample: Standard solutions in H_2O (100 $\mu g\ ml^{-1}$)

Sample Size: 20 µl

Eluent: [70% CH_3COONa (0.3M): 30% MeOH with PDFOA added], 0.1% Et_3N, pH 4.5

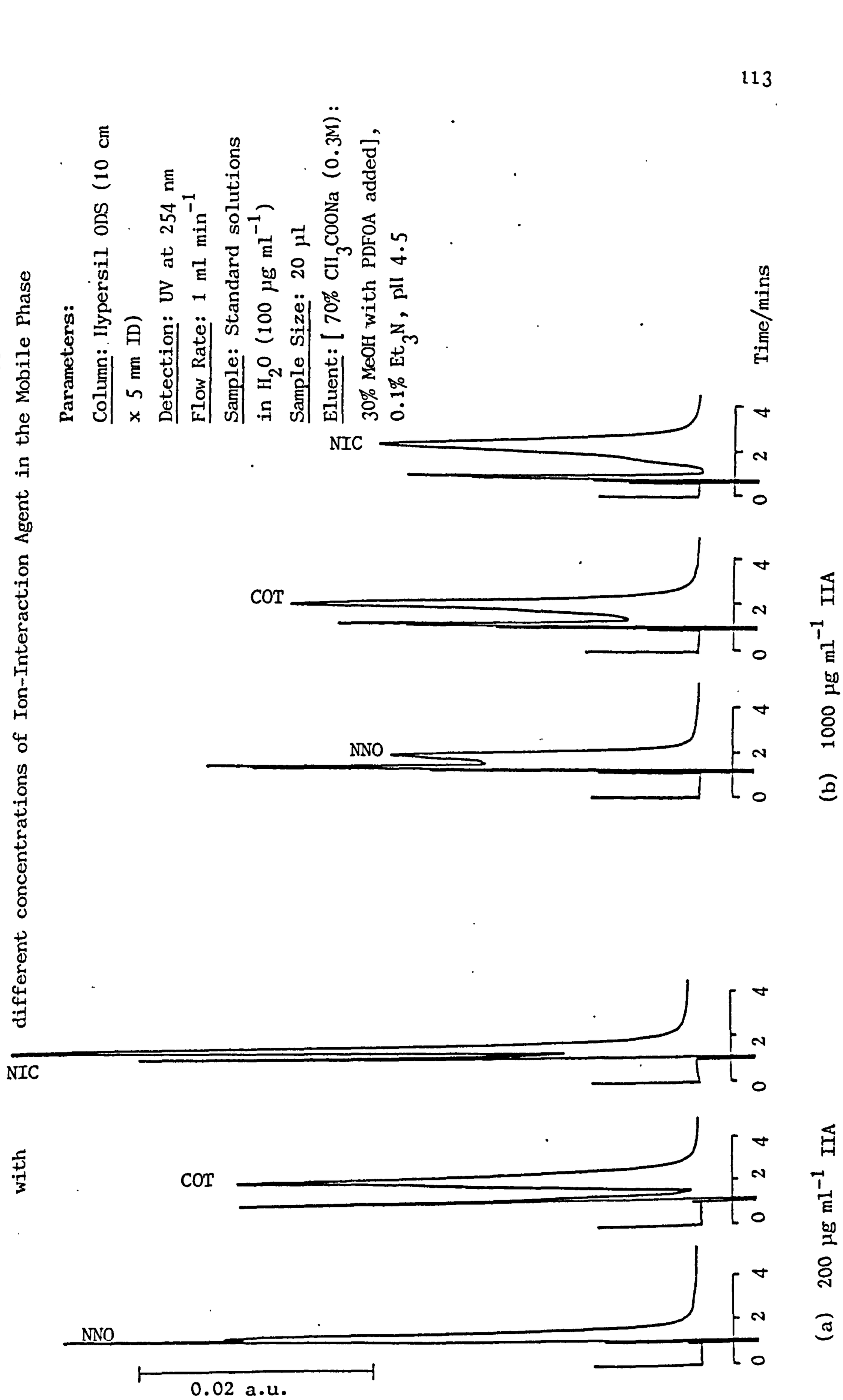


Table 2.4: The relationship between the retention time/capacity factor and the concentration of ion-interaction agent in the mobile phase for standard components by RP-IIC on a Hypersil ODS column

Concentration of IIA in the mobile phase ($\mu g\ ml^{-1}$)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
200	0.8	1.3	0.62	2.4	2.00	1.7	1.12
1000	0.8	1.8	1.25	2.0	1.50	2.3	1.88

Variation in the ratio of buffer to MeOH showed that as the percentage of MeOH was decreased the retention time increased; unfortunately the peak shape also deteriorated, see table 2.5.

Table 2.5: The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components by RP-IIC on a Hypersil ODS column

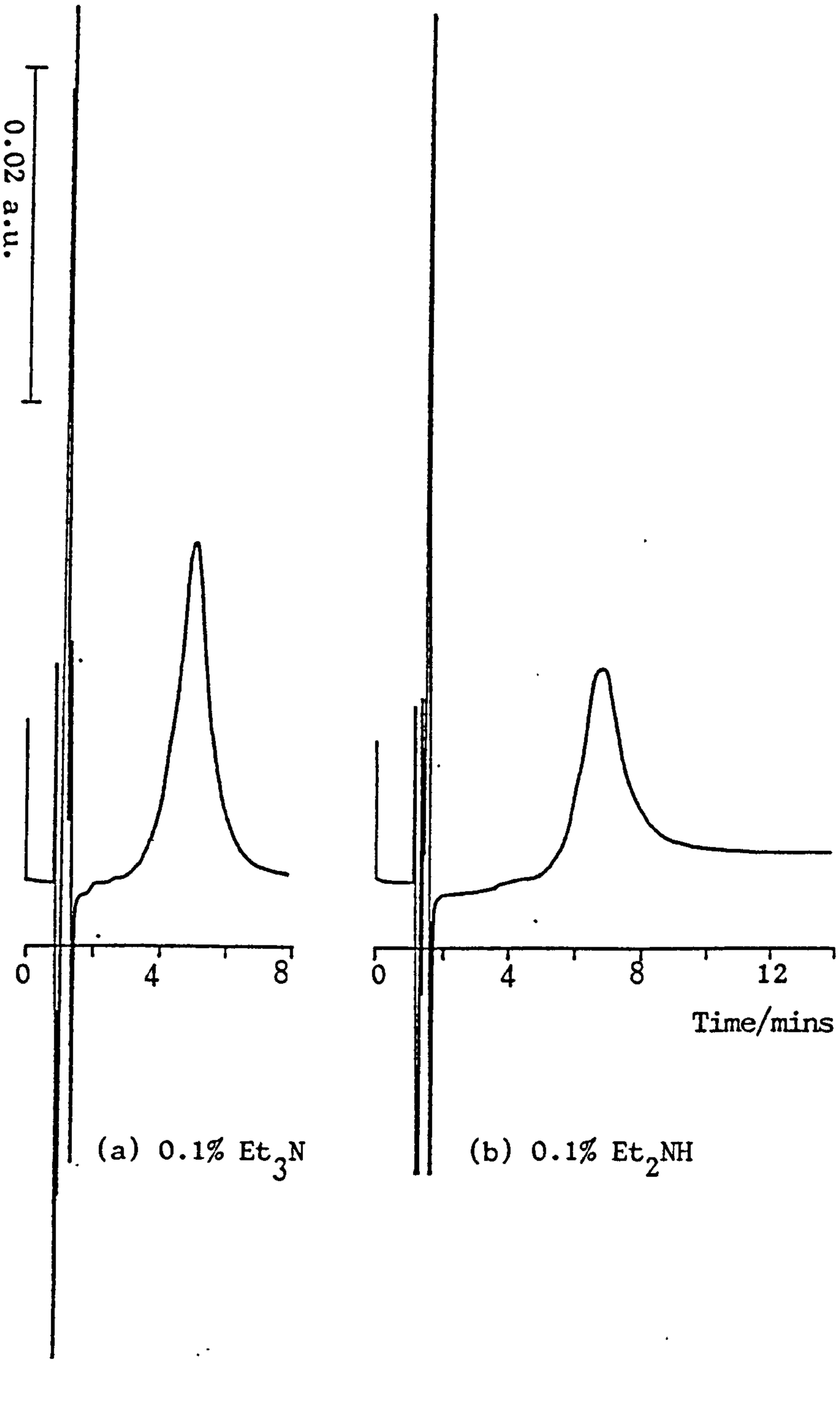
% Organic Modifier	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
10	0.8	9.2	10.50	5.1	5.38	8.2	9.25
20	1.8	8.3	3.61	5.0	1.78	10.5	4.83
30	0.8	1.8	1.25	2.0	1.50	2.3	1.88

With CH_3COONa 0.3M, pH 4.5 : 1000 $\mu g\ ml^{-1}$ PDFOA in MeOH (90:10), Et_2NH was substituted for Et_3N resulting in increased retention times for the standard components and also peak tailing, hence use of Et_3N was continued, see figure 2.22.

Figure 2.22: Effect of the Addition of Et_3N or Et_2NH (0.1%) to the Mobile Phase on the Chromatography of a Cotinine Standard (100 $\mu g\ ml^{-1}$)

Parameters: see figure 2.21, except for

Eluent: [90% CH_3COONa (0.3M): 10% MeOH with PDFOA (1000 $\mu g\ ml^{-1}$) added], pH 4.55, 0.1% Et_3N or Et_2NH, as stated below.



(a) 0.1% Et_3N

(b) 0.1% Et_2NH

Again with a mobile phase composition of 90% buffer to 10% MeOH, the pH was changed from pH 4.5 to pH 3.6. The retention times of the three standards were reduced dramatically with cotinine being virtually unretained. Adjustment to an intermediate pH of 4.1 increased the retention times of all three components but also caused the peak shape to become broad and tailing, as shown in table 2.6 and figure 2.23. Separation of the three standards was not forthcoming under any of the conditions tried and so the Hypersil column was abandoned.

Table 2.6: The relationship between the retention time/capacity factor and pH of the mobile phase for standard components by RP-IIC on a Hypersil ODS column

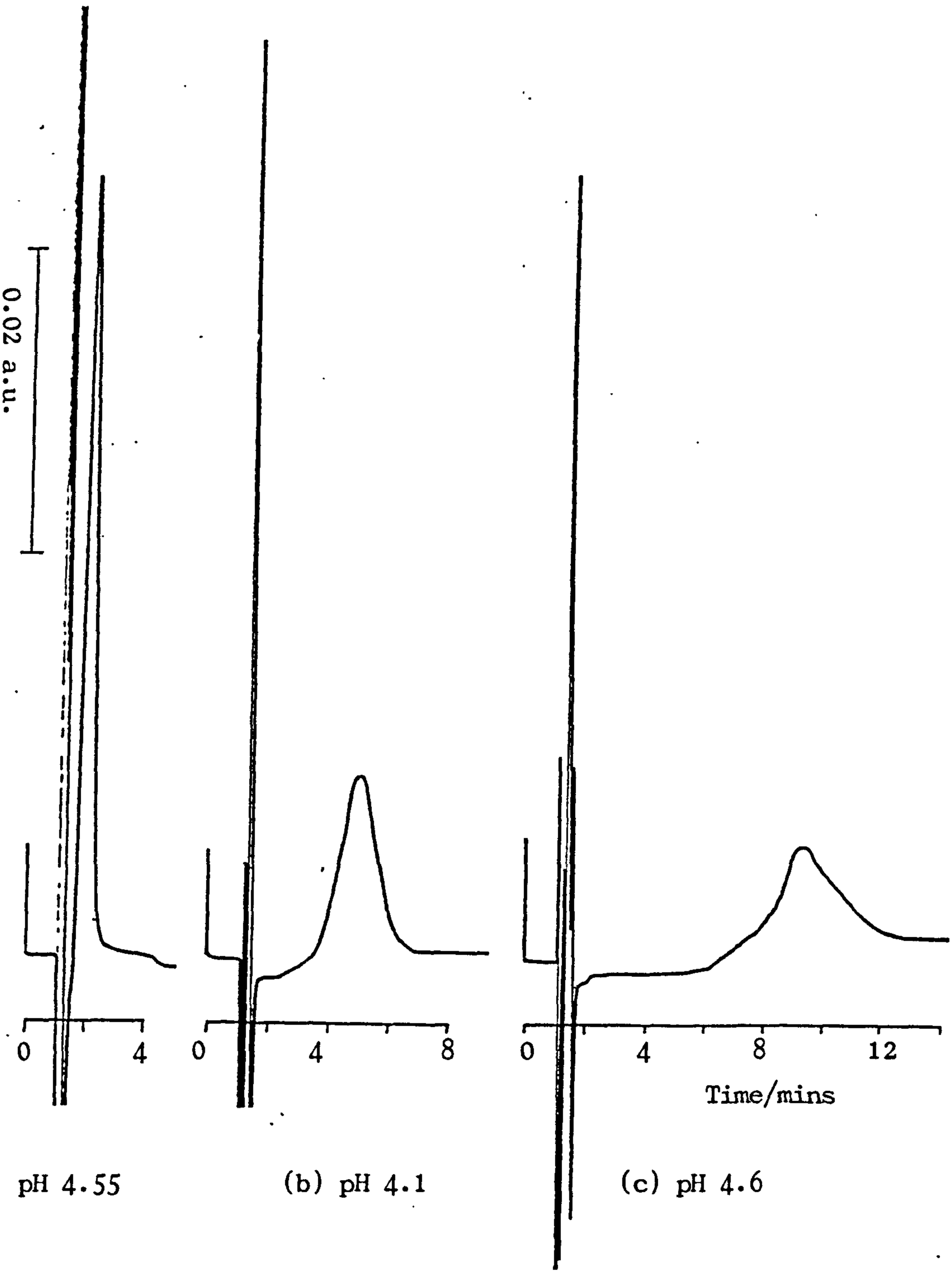
pH	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
3.6	0.8	2.0	1.50	1.8	1.25	2.0	1.50
4.1	1.0	5.1	4.10	4.0	3.00	4.7	3.70
4.5	0.8	9.2	10.50	5.1	5.38	8.2	9.25

On a Spherisorb ODS 5 µ column (10 cm x 5 mm ID) with a mobile phase of CH_3COONa, 0.3M, pH 4.5 : 1000 µg ml^{-1} PDFOA in MeOH, 70:30, nicotine, cotinine and nicotine-1'-N-oxide were all more strongly retained than on the Hypersil ODS column. The chromatography was most unsatisfactory and separation of the standards was not possible under the conditions employed. Changing only the pH from 4.5 to 3.5 resulted in a shortening of the retention times of all standards as was the case on the Hypersil columns. As the standards were now

Figure 2.23: Effect of Mobile Phase pH on the Chromatography of a Nicotine-1'-N-oxide standard (100 $\mu g\ ml^{-1}$)

Parameters: See figure 2.21 except for:

Eluent: [90% CH_3COONa (0.3M): 10% MeOH with PDFOA (1000 $\mu g\ ml^{-1}$) added], pH as stated below, 0.1% Et_3N.



(a) pH 4.55 (b) pH 4.1 (c) pH 4.6

all virtually unretained but the peak shape was satisfactory, increased retention was brought about by changing the ratio of buffer to methanol from 70:30 to 80:20 and finally to 95:5. Unfortunately the peak shape of both nicotine and nicotine-1'-N-oxide deteriorated, especially when the 95:5 ratio was employed, and again no separation of the standards was possible.

A change to a μ-Bondapak ODS column (30 cm x 3.9 mm ID) resulted in a separation of nicotine and nicotine-1'-N-oxide from cotinine, with nicotine and nicotine-1'-N-oxide co-eluting. The separation was achieved using an eluent of CH_3COONa, 0.3M, pH 4.5 : 1000 μg ml^{-1} PDFOA in MeOH 70:30, 0.1% Et_3N and is illustrated in figure 2.24 with retention times/capacity factors reported in table 2.7.

Table 2.7: The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components, by RP-IIC on a μ-Bondapak ODS column

% Organic Modifier	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
30	1.4	4.1	1.93	3.4	1.43	4.6	2.29

In an attempt to resolve nicotine and nicotine-1'-N-oxide the ratio of buffer to methanol was varied from 70:30 to 85:15 and finally to 80:20; however, nicotine and nicotine-1'-N-oxide co-eluted on every occasion and showed no sign of being resolved into two peaks, as illustrated by figure 2.25 and table 2.8.

Figure 2.24: Reverse Phase Ion-Interaction Chromatography of Individual Standards on a μ-Bondapak ODS column

Parameters:

Column: μ-Bondapak ODS (30 cm x 3.9 mm ID)
Detection: UV at 254 nm Flow rate: 2 ml min^{-1}
Sample: Individual standard solutions in H_2O (100 μg ml^{-1}) and a standard mixture in H_2O (50 μg ml^{-1})
Sample Size: 20 μl
Eluent: [70% CH_3COONa (0.3M): 30% MeOH with PDFOA (1000 μg ml^{-1}) added], pH 4.5, 0.1% Et_3N

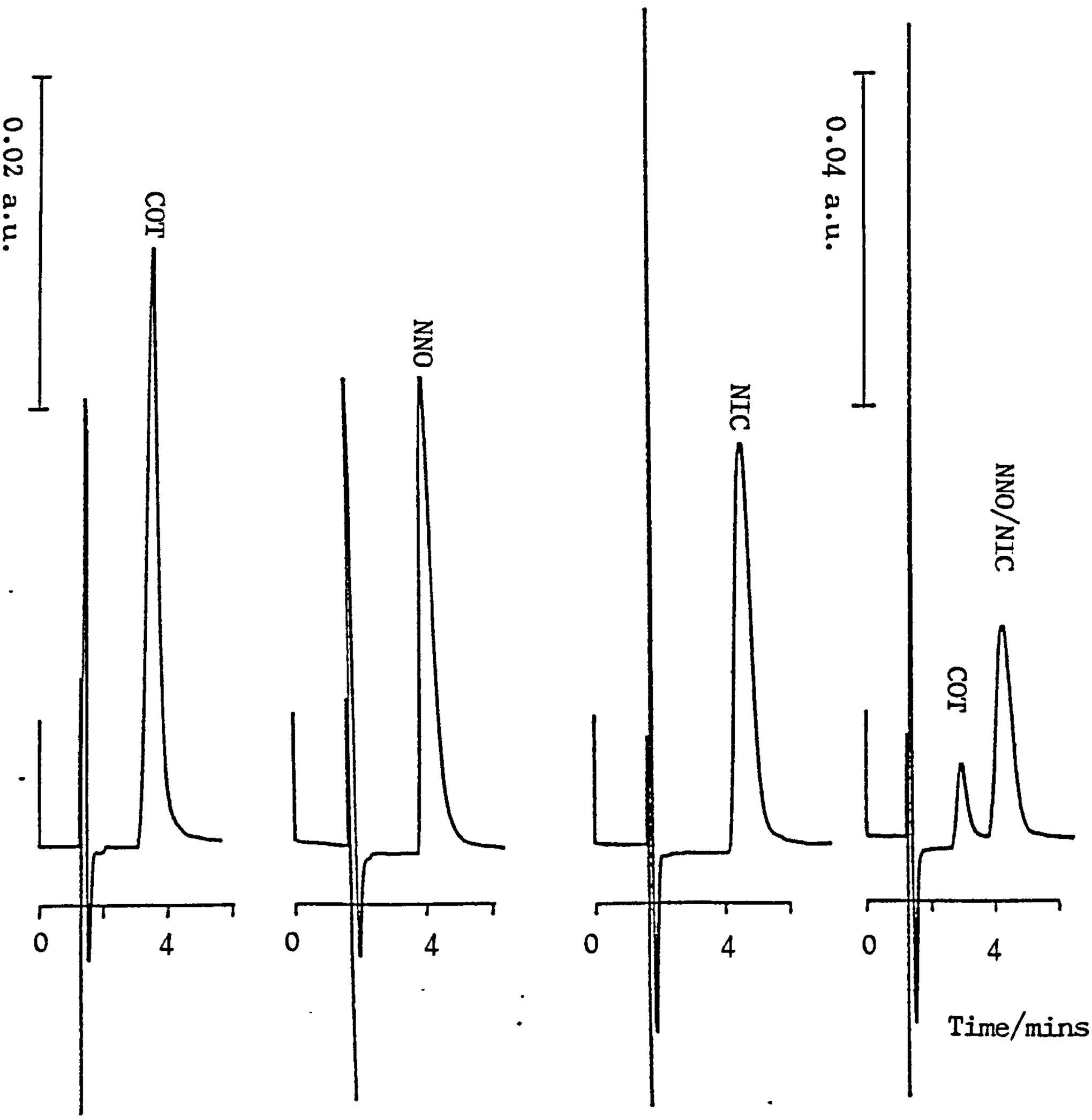


Figure 2.25: Effect of Mobile Phase Composition on the Reverse Phase Ion-Interaction Chromatography of a Standard Mixture on a μ-Bondapak ODS column

Parameters: see figure 2.24 except for: Flow Rate: 2.5 ml min^{-1}

Eluent : (a) [80% CH_3COONa (0.3M): 20% MeOH with PDFOA (1000 μg ml^{-1}) added], pH 4.5, 0.1% Et_3N

(b) [85% CH_3COONa (0.3M): 15% MeOH with PDFOA (1000 μg ml^{-1}) added], pH 4.5, 0.1% Et_3N

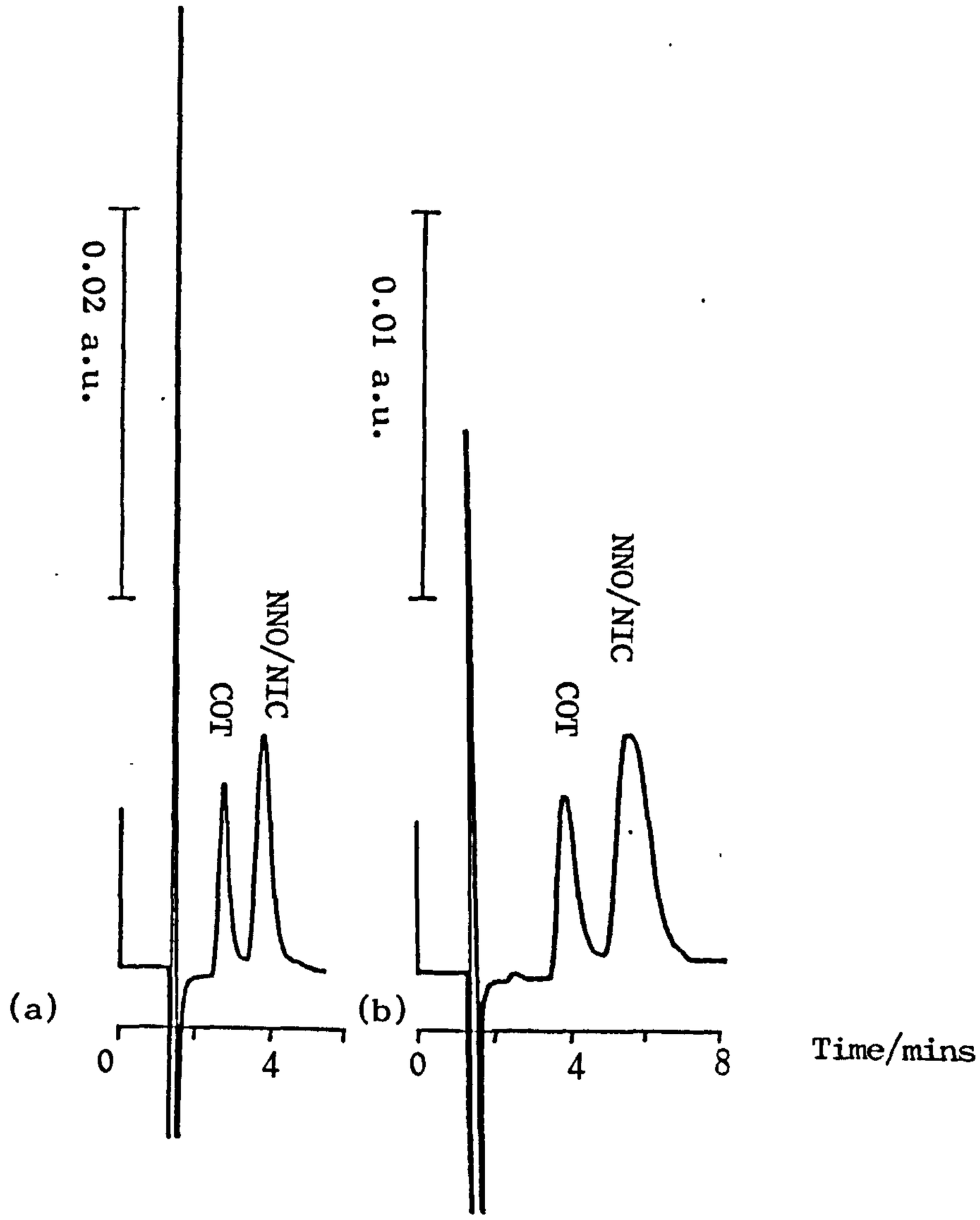


Table 2.8: The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components by RP-IIC on a μ-Bondapak ODS column

% Organic Modifier	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
15	1.2	5.5	3.58	3.8	2.17	5.3	3.42
20	1.3	4.1	2.15	2.8	1.15	4.1	2.15

The separations attempted, all based on ionic compounds, were difficult to control and from these results both the IC and RP-IIC modes were presumed unlikely to yield a satisfactory separation, therefore attention was focused instead on partition chromatography working with neutral alkaloids at a pH of 7 or above, as described by Saunders and Blume.[196]

2.9.2 Reverse Phase Partition Chromatography

In 1981 Saunders and Blume[196] published a HPLC method for the quantitation of the major alkaloids, anatabine, anabasine, nicotine and nornicotine, found in both fresh green leaf and air dried tobacco leaf samples. The tobacco alkaloids were separated successfully on a reversed-phase μ-Bondapak C_{18} column with an isocratic eluent of 0.2% phosphoric acid, pH adjusted to 7.25 with triethylamine: methanol (60:40).

This same method was used to chromatograph nicotine, nicotine-1'-N-oxide and cotinine. Several reversed-phase columns were examined

and these are listed in table 2.9. On the μ-Bondapak column (30 cm x 3.9 mm ID), a separation of nicotine and its major metabolites was achieved with a mobile phase containing 20% MeOH. Variation of the k' values of all the standards with changes in the methanol content of the eluent is shown in table 2.10 and figures 2.26 and 2.27. In all cases an increase in the percentage of the organic solvent MeOH caused a decrease in the k' value of the standard. Eluent strength is increased by increasing the solubility of the solute in the mobile phase usually brought about by decreasing its aqueous content.

Acetonitrile was substituted for methanol in the mobile phase. In both cases the trends in k' values, when the percentage of organic modifier was varied, were similar, see table 2.11 and figures 2.28 and 2.29. To obtain a k' value of 3.0 for cotinine required 20% MeOH but only 9% CH_3CN. This was in accordance with the general rule that the lower the polarity of the mobile phase, the higher is its eluent strength, acetonitrile being less polar than methanol.

On an R-Sil ODS column (25 cm x 5 mm ID), the relationships between % MeOH or % CH_3CN and k' values were similar to those obtained on the μ-Bondapak column. However, using 30% CH_3CN, although the nicotine-1'-N-oxide and cotinine were not resolved, it was noted that cotinine eluted before nicotine-1'-N-oxide, see figures 2.30-2.34 and tables 2.12 and 2.13.

As the separations obtained on both the μ-Bondapak and R-Sil columns showed no marked improvement when acetonitrile was employed in place of methanol, acetonitrile was no longer considered as an alternative for reasons of toxicity and cost.

TABLE 2.9: Specifications of stationary phases evaluated for the analysis of nicotine and its metabolites under reverse phase partition conditions

Stationary Phase	Manufacturer	Length (cm)	ID (mm)	dp (μm)	Particle Shape*	Carbon Loading (%)	Mean Pore Diameter (nm)	Surface Area $m^2\ g^{-1}$	Surface+ Coverage $g\ m^{-2}$	Degree of End-Capping	No. of Plates, N per metre
μ-Bondapak ODS	Waters Associates	30	3.9	10	I	10	12.5	300-500	0.33	End-capped	1333
Spherisorb ODS 1	Phase Separations Group	10	5	5	S	7	8	220	0.32	Partially end-capped	3000
Spherisorb ODS 2	Phase Separations Group	10	5	5	S	12	8	220	0.55	Fully end-capped	1960
R-Sil ODS	HPLC Technology	25	5	10	-	-	-	-	-	-	1664
PL-RP-S	Polymer Labs	15	4.6	5	-	-	10	550	-	-	1893
Partisil ODS 2	Whatman	25	4.6	10	I	15	8.5	350+	0.43	uncapped	1432
Hypersil phenyl	Shandon-Southern Products	15	5	5	S	5	-	-	-	-	5567
Nucleosil NO_2	Macherey-Nagel	10	5	5	S	-	10	300	-	-	6370
Resolve C_{18}	Waters Associates	15	5	5	S	12	9	200	0.6	uncapped	3840
Resolve C_{18}•	Waters Associates	10	8	5	S	12	9	200	0.6	uncapped	4940

* S = Spherical
I = Irregular

• Radial Pak cartridge in the RCM-100 unit

+ surface coverage $(g\ m^{-2}) = \dfrac{\text{C loading (\%)} \times 1000}{\text{surface area } (m^2 \times g^{-1}) \times 100}$

Table 2.10 and Figure 2.26:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a μ-Bondapak ODS column

Table 2.10

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
20	1.6	3.7	1.31	6.4	3.0	13.4	7.37
30	1.6	3.3	1.06	3.7	1.31	7.7	3.81
40	1.6	2.8	0.75	2.8	0.75	5.3	2.31

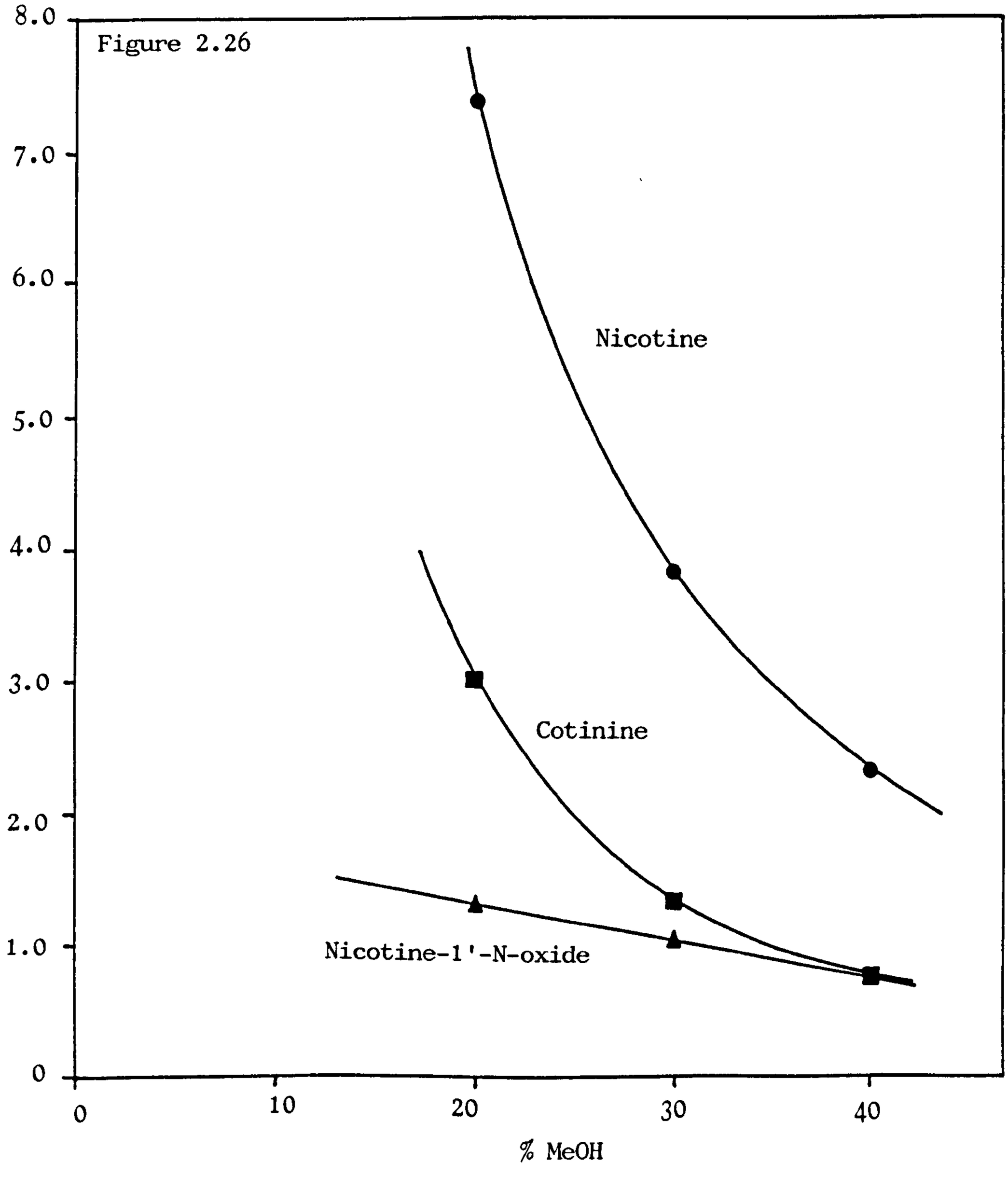


Figure 2.27: The Effect of Mobile Phase Composition (MeOH Content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a μ-Bondapak ODS column.

Parameters: Column: μ-Bondapak ODS (30 cm x 3.9 mm ID) Flow Rate: 2 ml min^{-1}

Detection: UV at 254 nm. Sample: Standard mixture in H_2O (10 μg ml^{-1})

Sample Size: 20 μl

Eluent: 0.2% H_3PO_4, pH adjusted to 7.25: MeOH in the ratio (a) 80:20, (b) 70:30 and (c) 60:40.

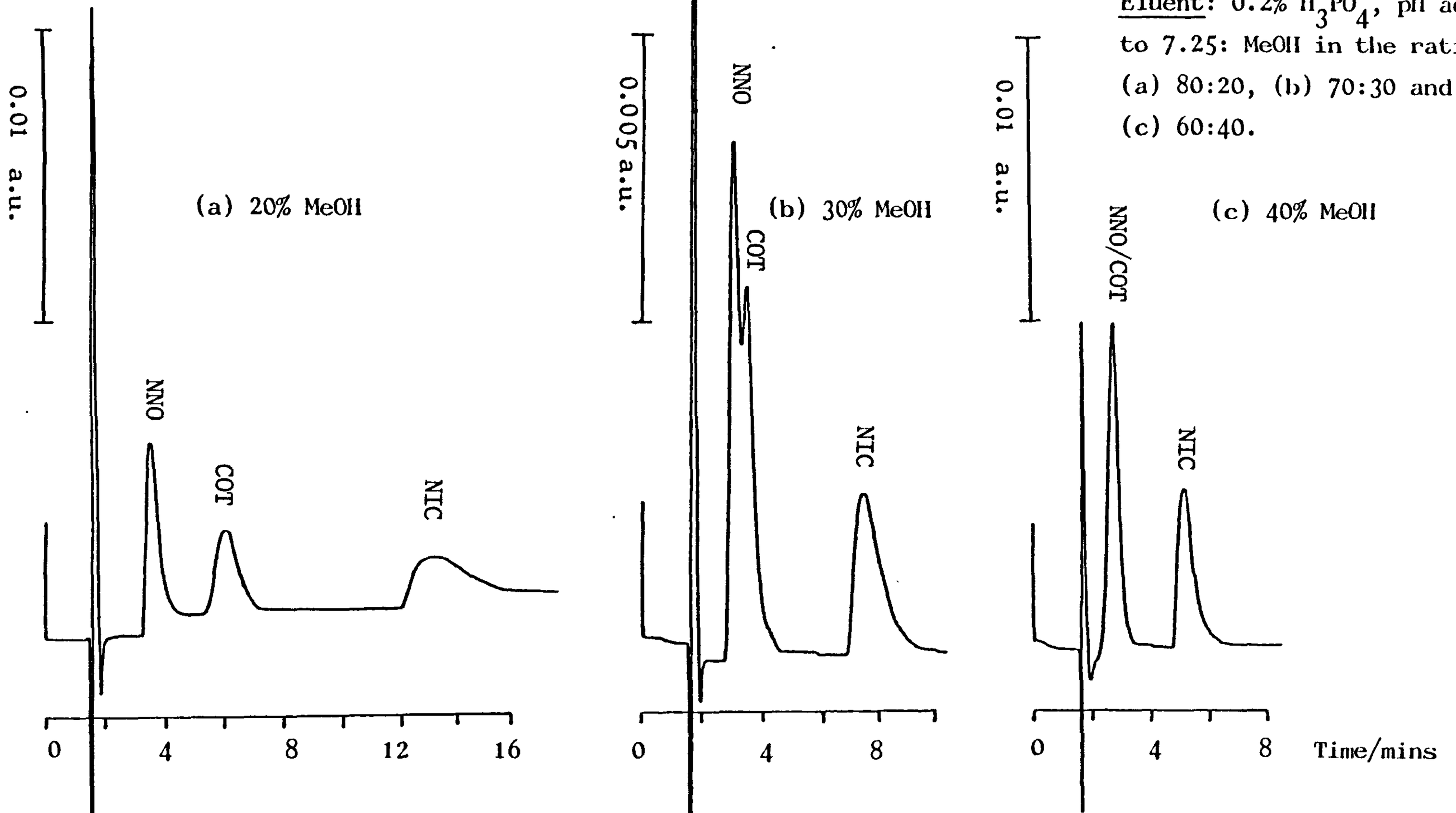


Table 2.11 and Figure 2.28:
The relationship between the retention time/capacity factor and acetonitrile content of the mobile phase for standard components under reverse phase partition conditions on a μ-Bondapak ODS column

Table 2.11

% Organic Modifier (CH_3CN)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
10	1.4	3.3	1.36	5.2	2.71	9.6	5.86
20	1.4	2.6	0.86	2.6	0.86	5.2	2.71

Figure 2.28

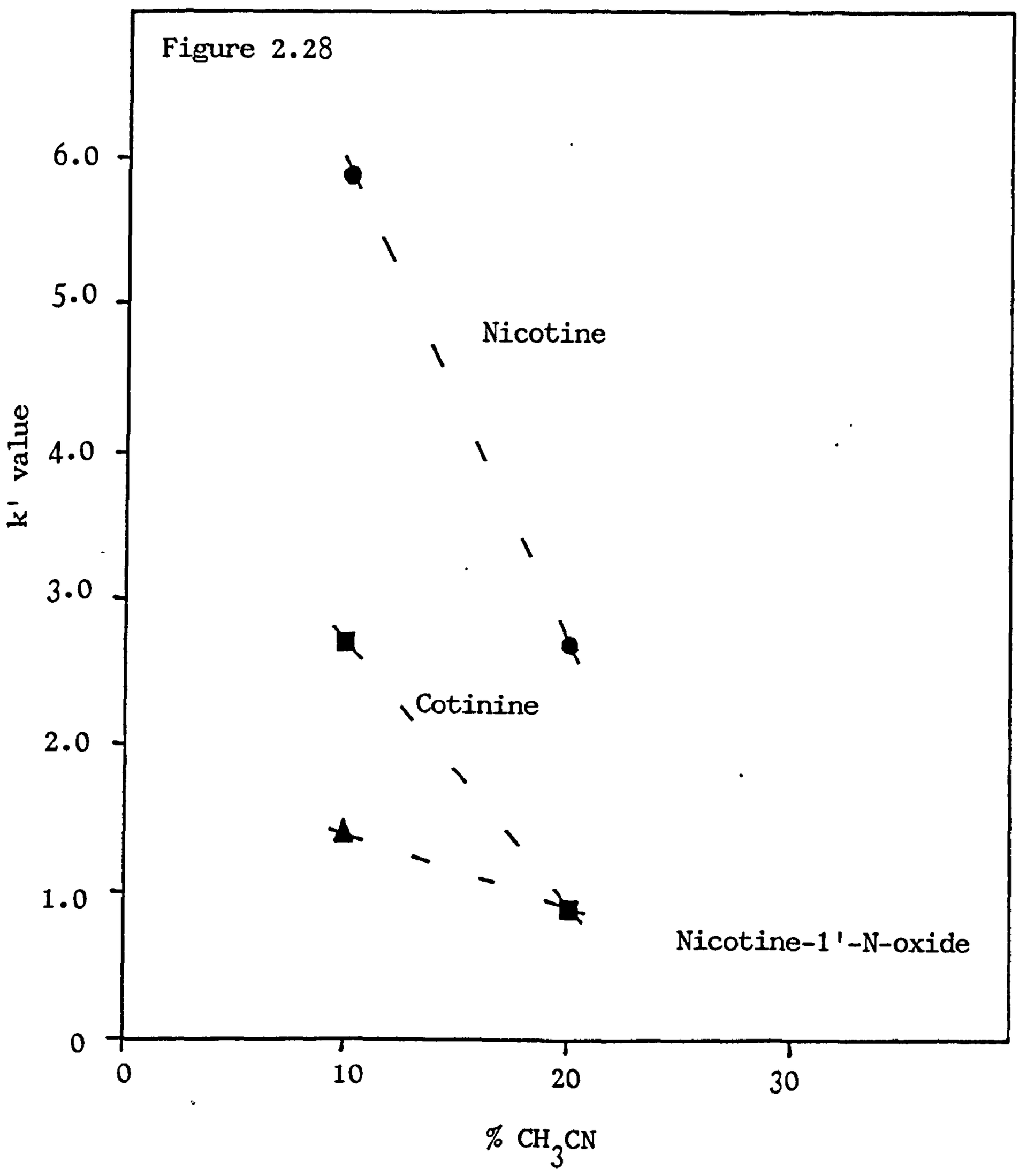


Figure 2.29: The Effect of Mobile Phase Composition (CH_3CN Content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a μ-Bondapak ODS column

Parameters: see figure 2.27 except for:

Detection: UV at 260 nm

Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:CH_3CN in the ratio (a) 90:10, and (b) 80:20

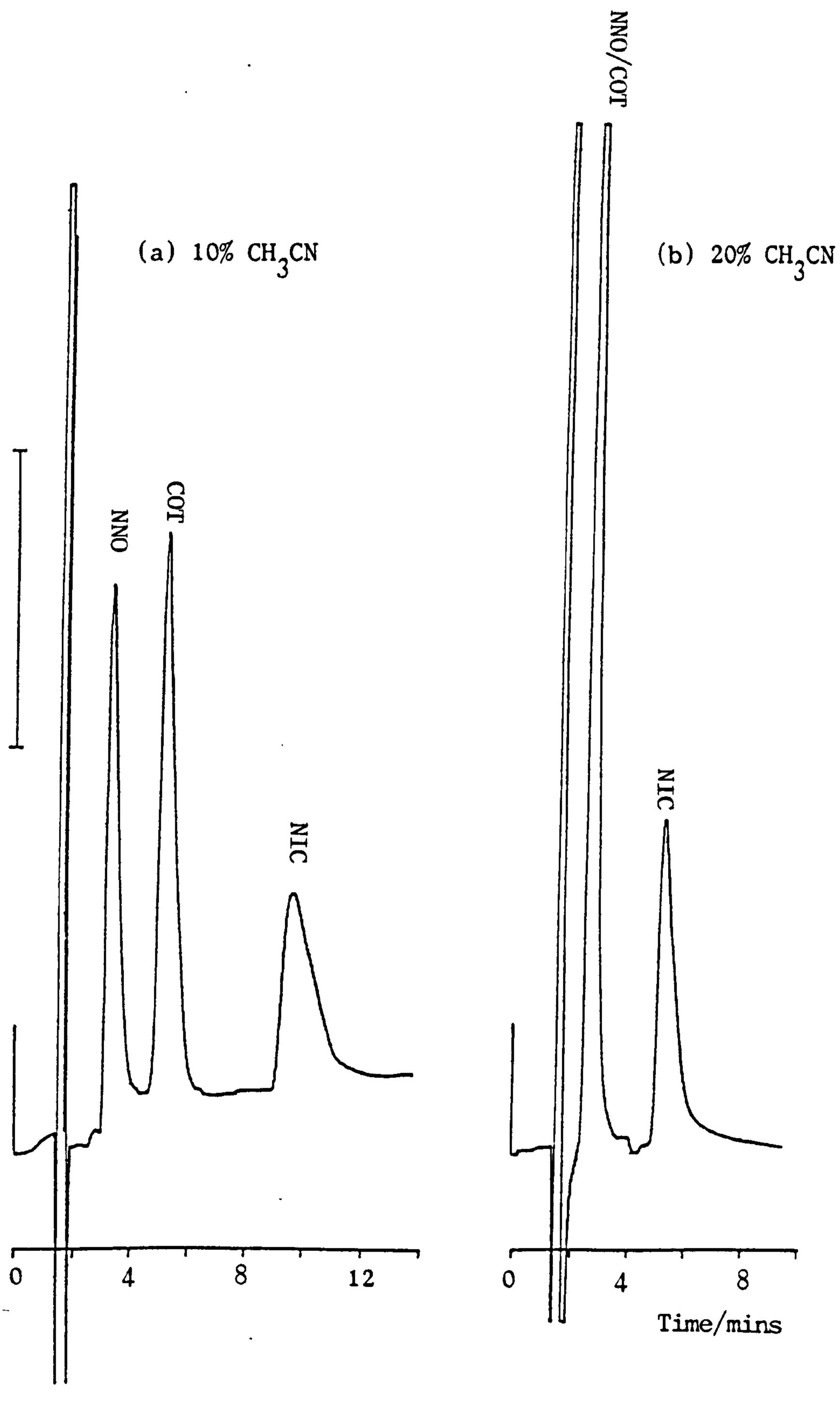


Table 2.12 and Figure 2.30:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on an R-Sil ODS column

Table 2.12

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
30	2.0	4.2	1.10	6.0	2.00	12.6	5.30
40	2.3	4.4	0.91	4.6	1.00	10.3	3.48

Figure 2.30

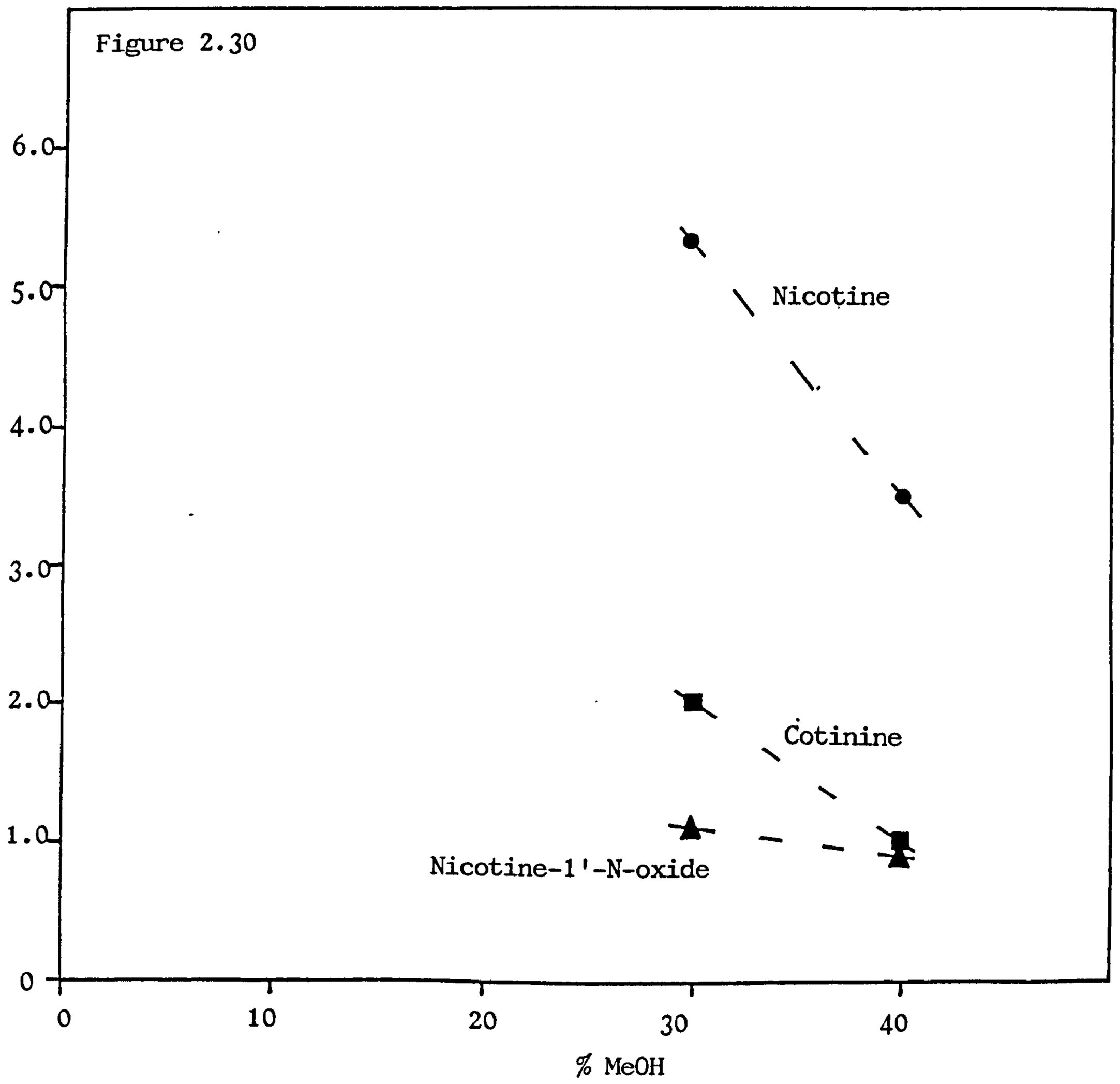


Figure 2.31: The Effect of Mobile Phase Composition (MeOH content) on the Reverse Phase Partition Chromatography of a Standard Mixture on an R-Sil ODS column

Parameters: Column: R-Sil ODS (25 cm x 5 mm ID)
Detection: Standard mixture in H_2O (10 μg ml^{-1})
Sample Size: 20 μl. Flow Rate: (a) 1.2 ml min^{-1}
(b) 1.5 ml min^{-1}
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH in the ratio (a) 60:40 and (b) 70:30

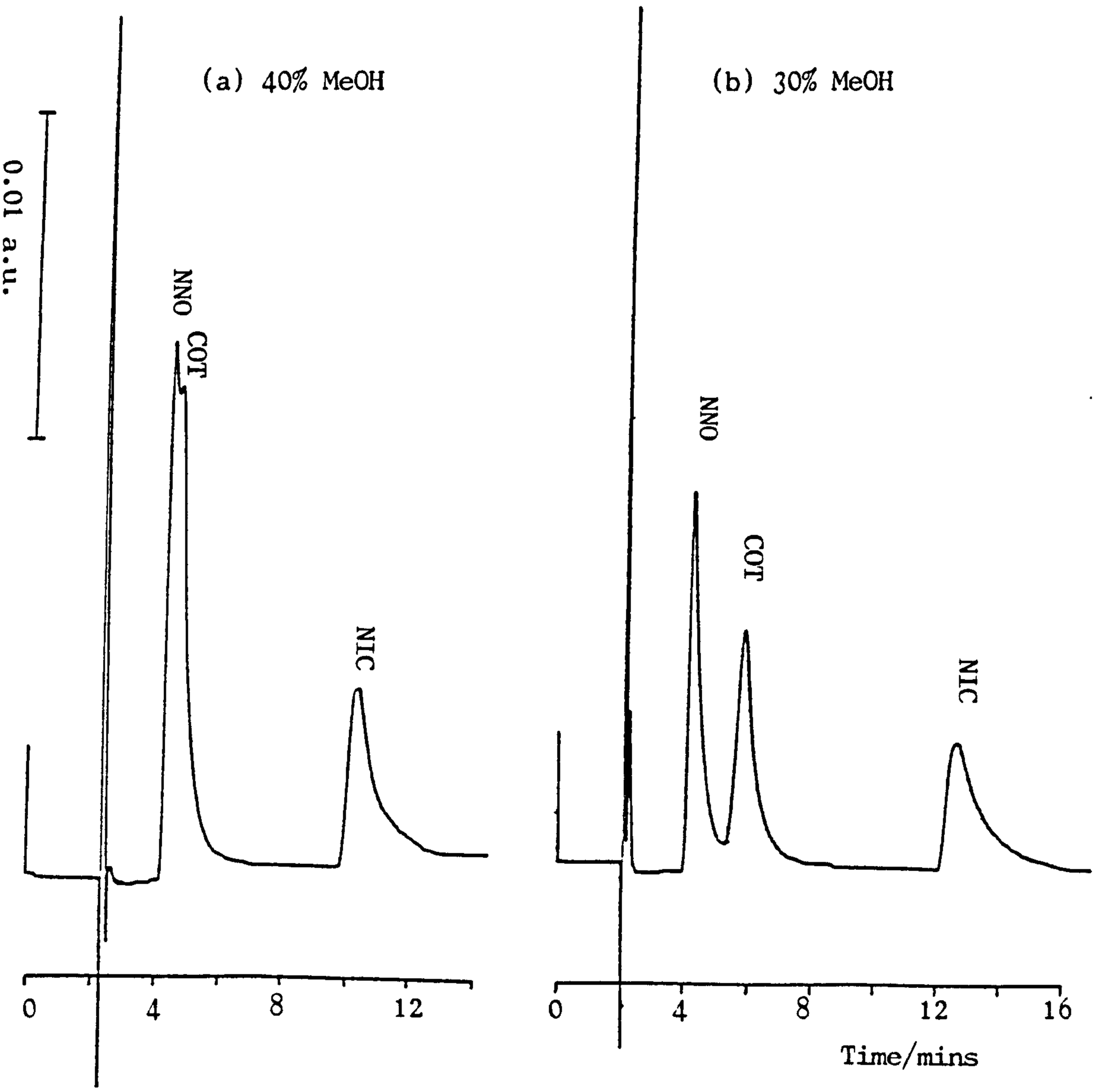


Table 2.13 and Figure 2.32:
The relationship between the retention time/capacity factor and acetonitrile content of the mobile phase for standard components under reverse phase partition conditions on an R-Sil ODS column

Table 2.13

Organic Modifier (CH_3CN)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
10	2.0	4.9	1.45	10.3	4.15	16.6	7.30
20	1.8	3.6	1.00	4.4	1.44	7.6	3.22
30	1.6	3.2	1.00	2.9	0.81	5.4	2.38

Figure 2.32

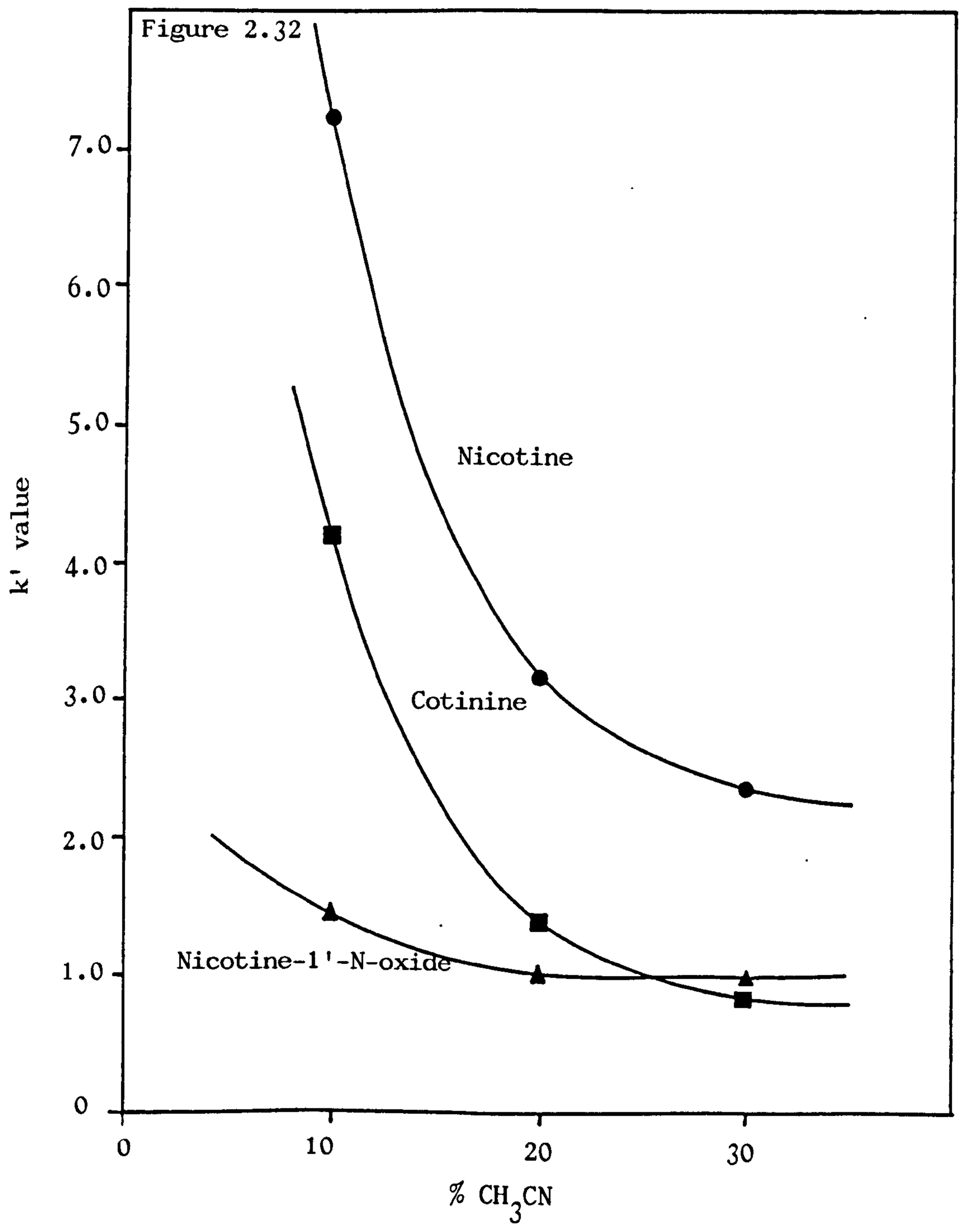


Figure 2.33: The Effect of Mobile Phase Composition (CH_3CN content) on the Reverse Phase Partition Chromatography of a Standard Mixture on an R-Sil ODS column

Parameters: see figure 2.31 except for: Flow Rate: 1.5 ml min^{-1}

Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N : CH_3CN (a) 70:30, (b) 80:20 and (c) 90:10.

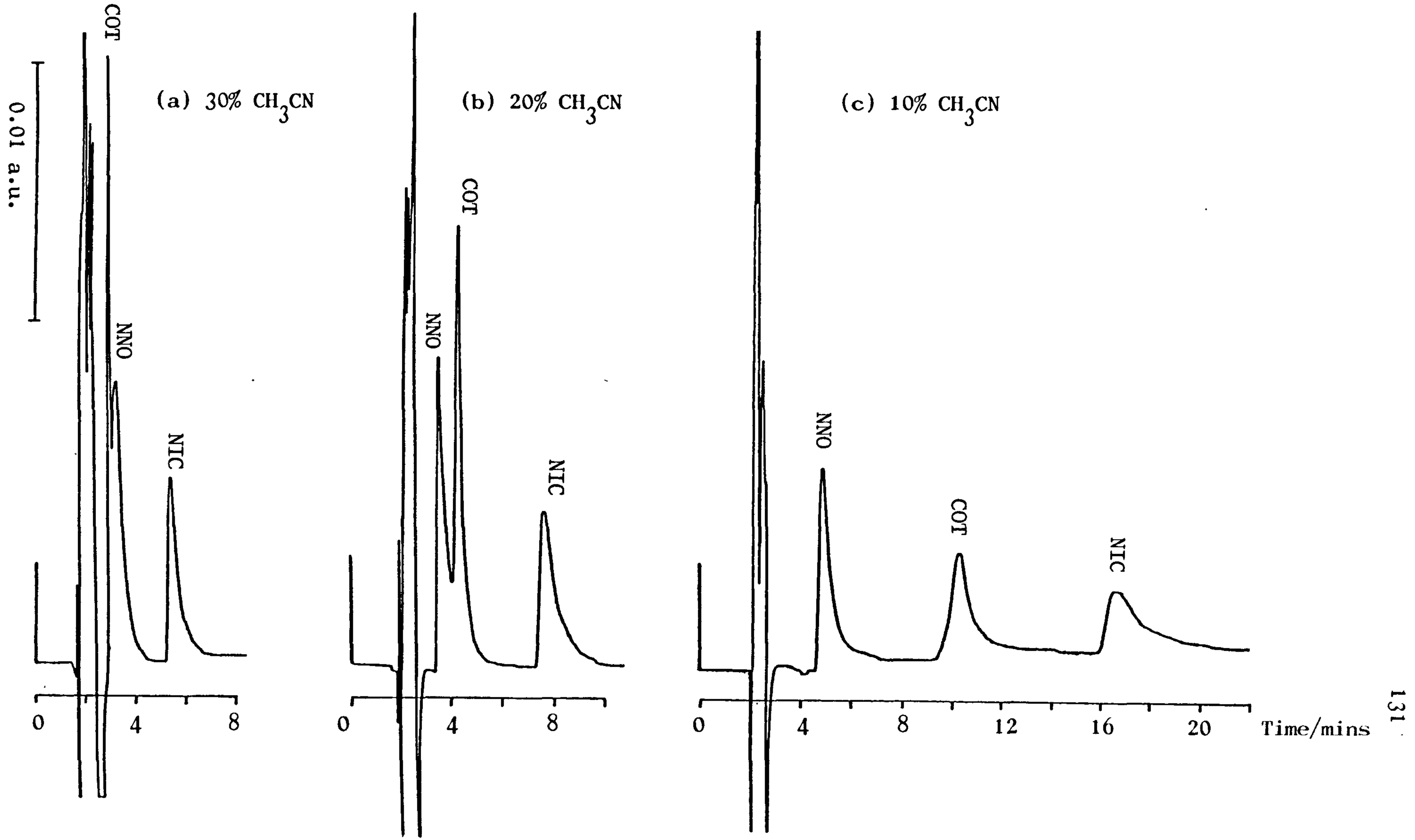


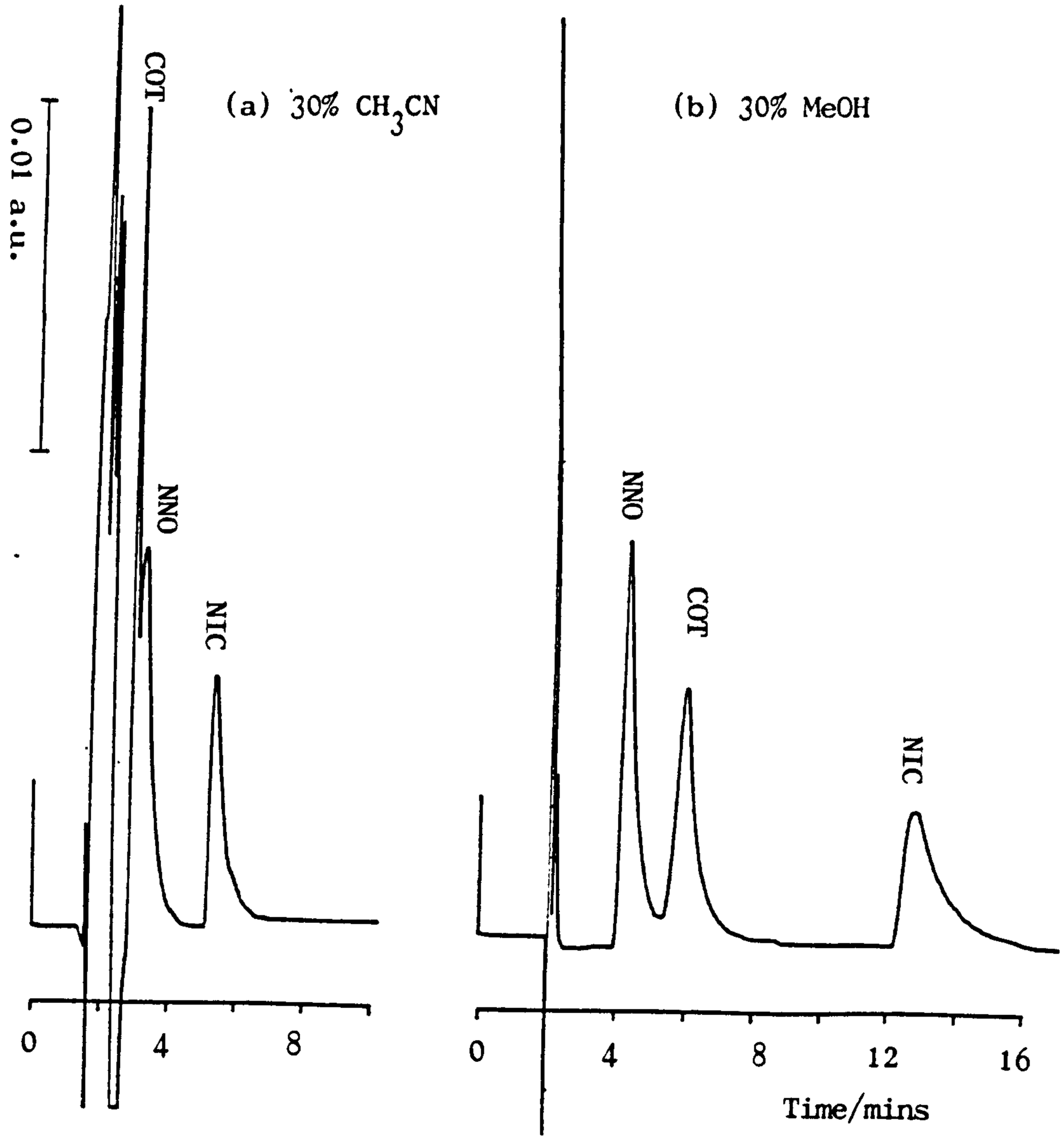
Figure 2.34: Comparison of the Reverse Phase Partition Chromatography on an R-Sil ODS column using (a) CH_3CN and (b) MeOH as the organic modifier in the Mobile Phase

Parameters: see figure 2.31, except for:

Flow Rate: 1.5 ml min^{-1}

Eluent: (a) 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N: CH_3CN (70:30)

(b) 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N: MeOH (70:30)



Other ODS columns examined which gave similar results to those already discussed included Spherisorb ODS1 (10 cm x 5 mm ID) and Spherisorb ODS2 (10 cm x 5 mm ID), see figures 2.35-2.38 and tables 2.14 and 2.15.

The elution order on all the columns mentioned above (using methanol as the organic modifier) was: nicotine-1'-N-oxide, followed by cotinine and lastly nicotine. The percentage of methanol which gave the best resolution between the three standards usually resulted in nicotine having a long retention time and so this peak was sometimes broad and tailing. An attempt was made to improve the peak shape by adding n-heptanol, nitromethane or pelargononitrile etc. to the methanol. The mobile phase then consisted of e.g. 1000 $\mu g\ ml^{-1}$ n-heptanol in methanol, 0.2% phosphoric acid, pH adjusted to 7.25 with Et_3N in the ratio 30:70. Two columns were used in this trial, μ-Bondapak and Spherisorb ODS1, the k' values are reported in tables 2.16 and 2.17. See also figures 2.39-2.41.

Although the peak shape was improved slightly by the use of some of these additives, e.g. n-heptanol, the improvement was probably mostly due to shortened retention times. Shorter retention times of all the standards was accompanied by a deterioration in the resolution of nicotine-1'-N-oxide and cotinine in particular. Therefore the use of additives was rejected.

Table 2.14 and Figure 2.35:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Spherisorb ODS1 column

Table 2.14

Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
20	0.4	1.8	3.50	5.2	12.00	14.3	34.75
30	0.5	1.5	2.00	3.1	5.20	8.0	15.00
40	0.5	1.2	1.40	1.6	2.20	4.3	7.60

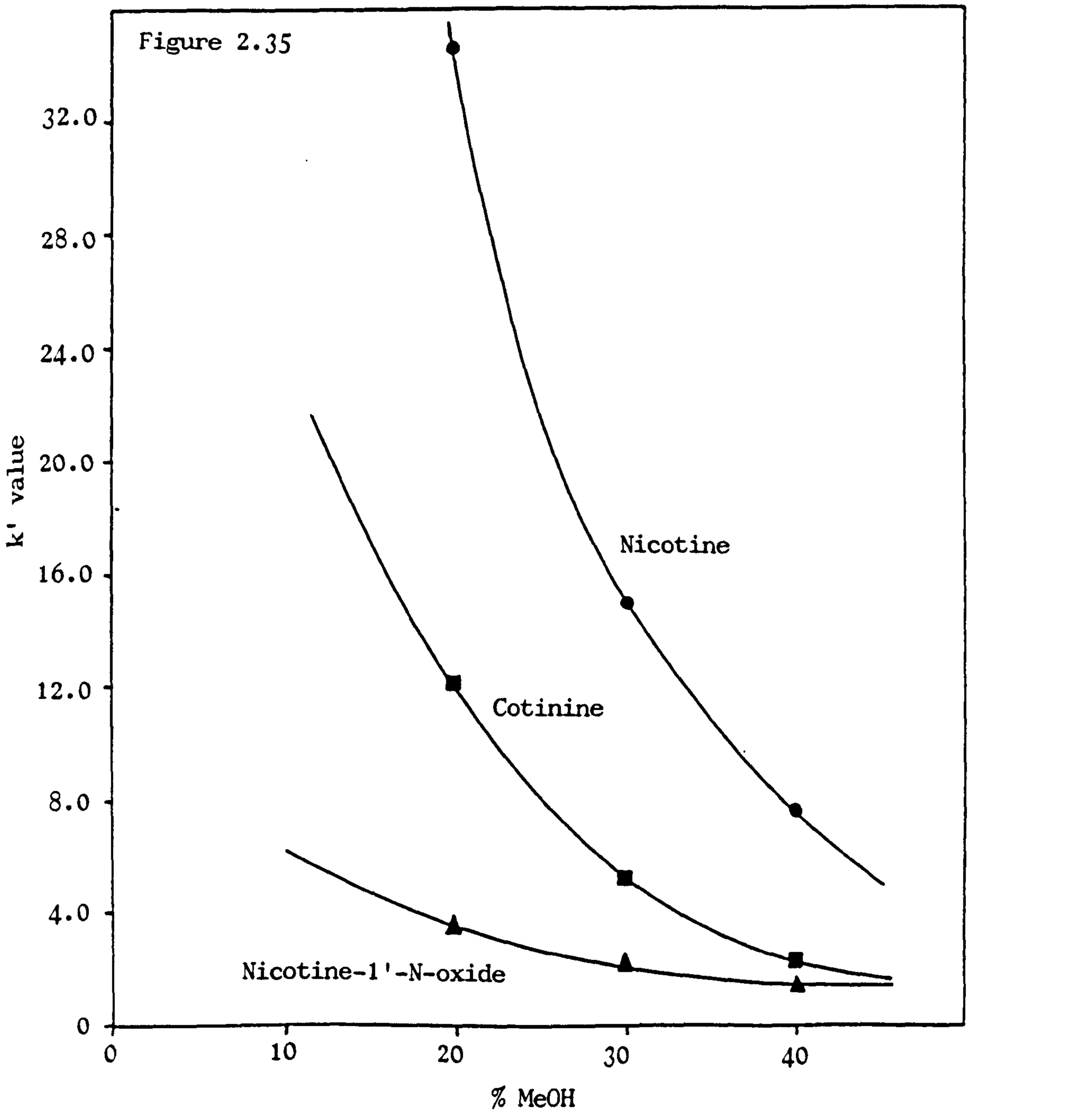


Figure 2.36: The Effect of Mobile Phase Composition (MeOH content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a Spherisorb ODS1 column

Parameters: Column: Spherisorb ODS1 (10 cm x 5 mm ID). Detection: UV at 260 nm
Sample: Standard mixture in H_2O (10 μg ml^{-1}). Sample Size: 20 μl
Flow rate: (a) 2 ml min^{-1}
(b) 2 ml min^{-1} and (c) 1 ml min^{-1}
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH in the ratio
(a) 60:40, (b) 70:30 and (c) 80:20

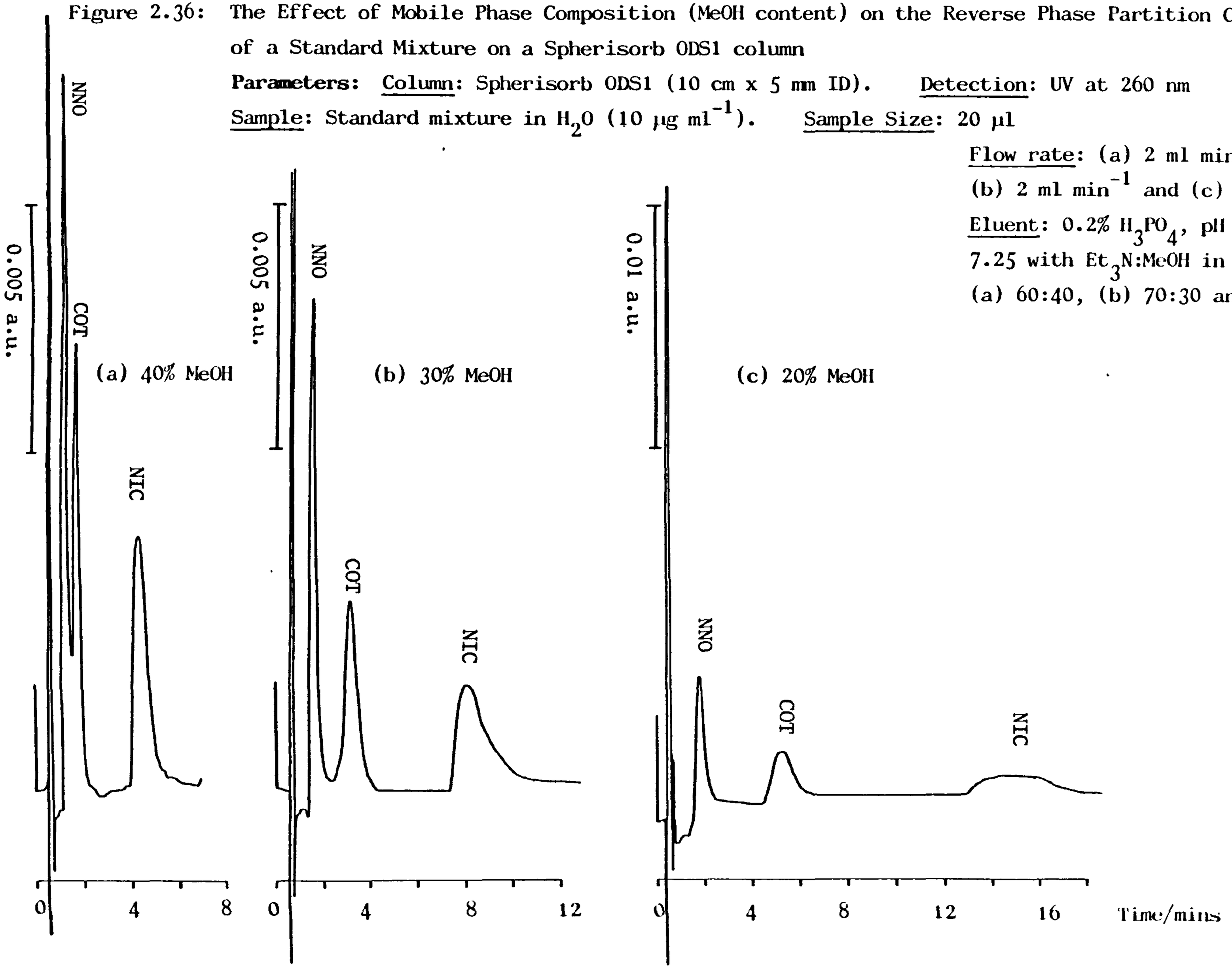


Table 2.15 and Figure 2.37:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Spherisorb ODS2 column

Table 2.15

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
20	0.4	1.0	1.50	3.2	7.00	5.9	13.75
30	0.6	1.2	1.00	2.0	2.33	5.7	8.50
40	0.4	0.9	1.25	1.0	1.50	1.9	3.75

Figure 2.37

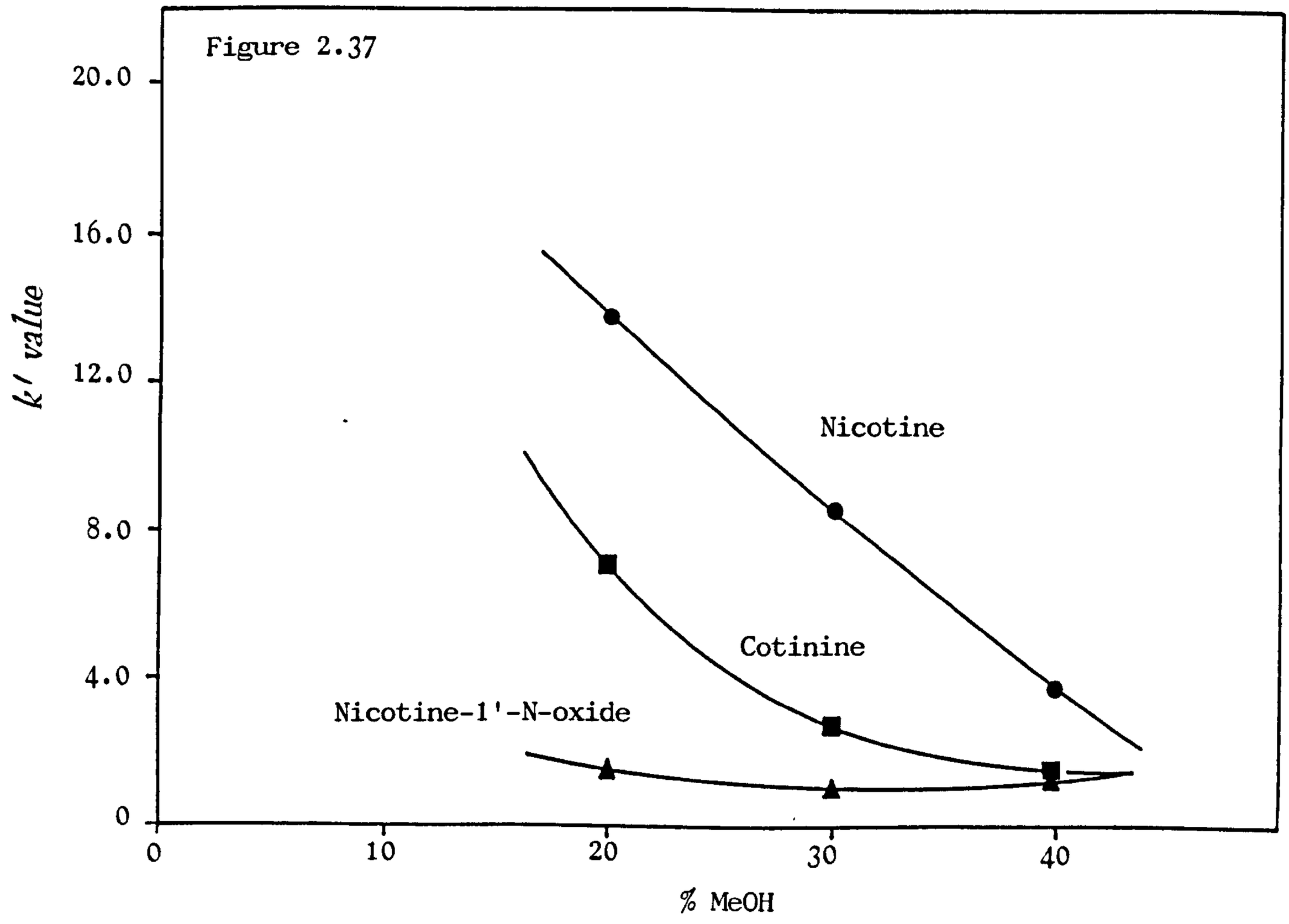


Figure 2.38: The Effect of Mobile Phase Composition (MeOH content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a Spherisorb ODS2 column

Parameters: Column: Spherisorb ODS2 (10 cm x 5 mm ID) Flow Rate: 2 ml min^{-1}. Detection: UV at 260 nm Sample: Standard mixture in H_2O (10 µg ml^{-1}) Sample Size: 20 µl. Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N: MeOH in the ratio (a) 60:40, (b) 70:30 and (c) 80:20

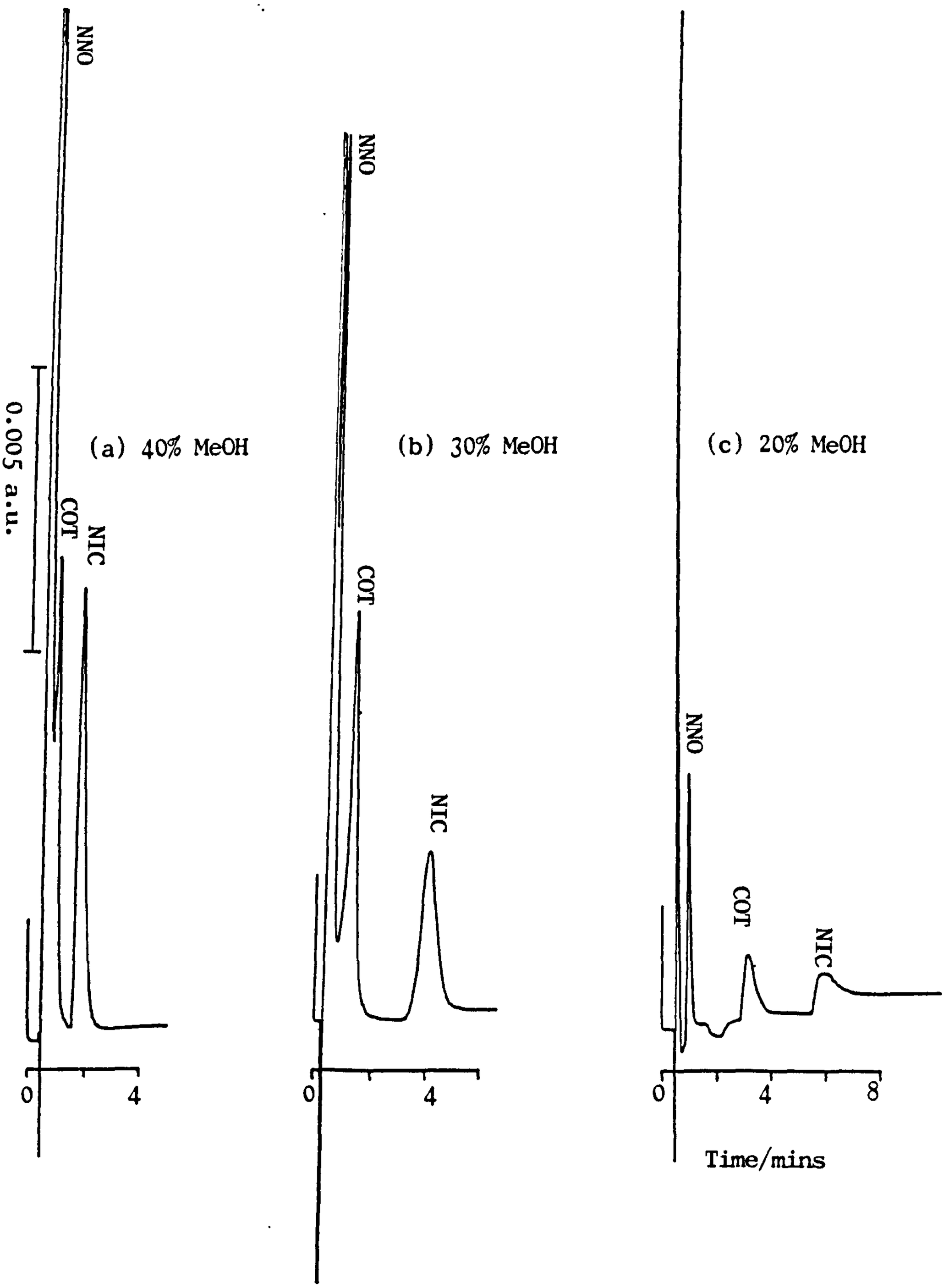


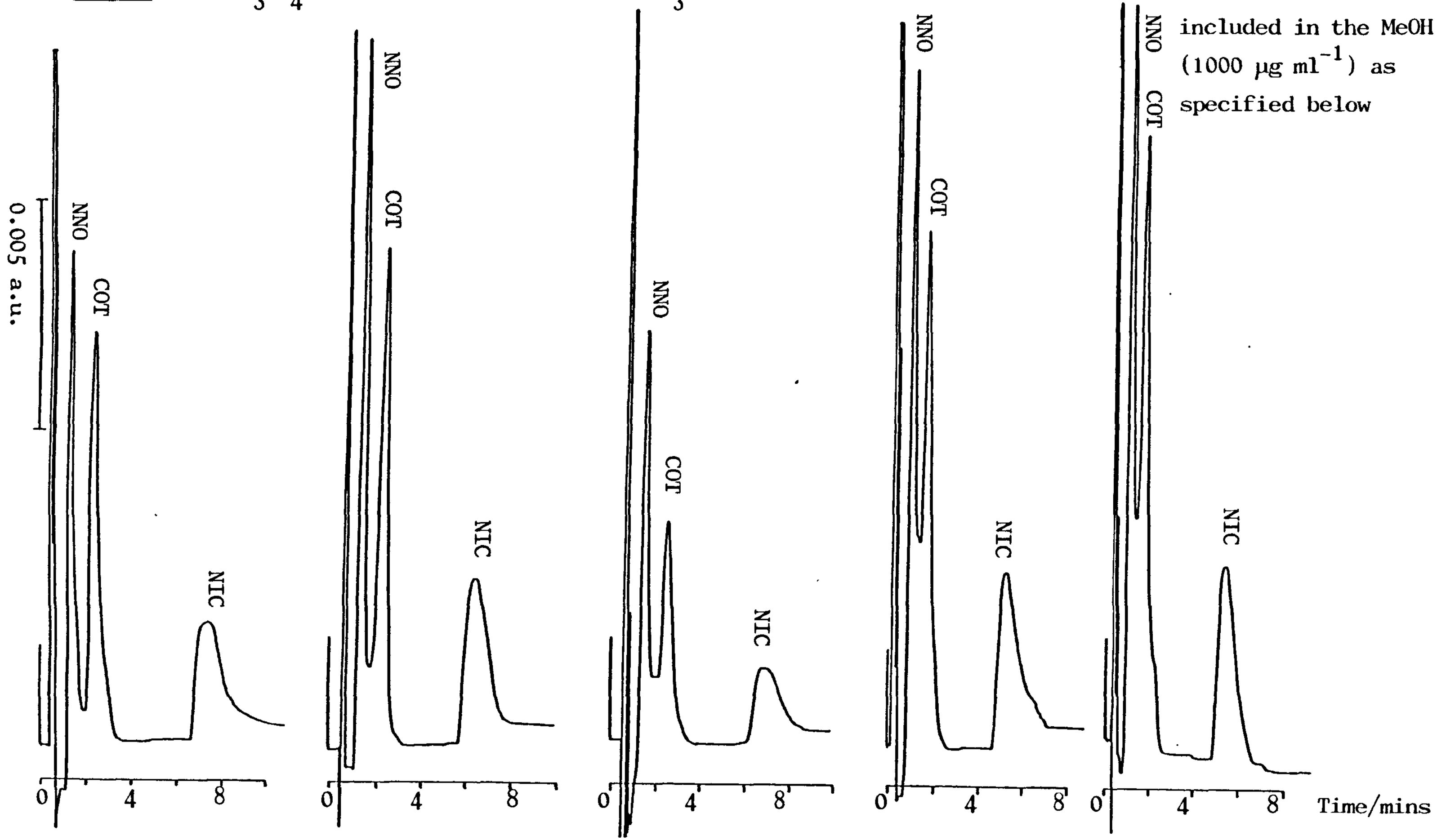
Table 2.16: The relationship between the retention time/capacity factor and a mobile phase containing 30% organic modifier plus different additives (1000 $\mu g\ ml^{-1}$) for standard components under reverse phase partition conditions on a Spherisorb ODS1 column

Additive	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
none	0.4	1.3	2.25	2.4	5.00	7.4	17.50
n-heptanol	0.5	1.4	1.80	2.3	3.60	6.5	12.00
CH_3NO_2	0.5	1.4	1.80	2.5	4.00	6.8	12.60
pelargono nitrile	0.2	1.1	4.50	1.7	7.50	5.3	25.50
n-octanol	0.3	1.2	3.0	1.7	4.67	5.3	16.67

Table 2.17: The relationship between the retention time/capacity factor and a mobile phase containing 30% organic modifier plus different additives (1000 $\mu g\ ml^{-1}$) for standard components under reverse phase partition conditions on a μ-Bondapak ODS column

Additive	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
none	1.5	3.2	1.13	3.6	1.40	7.7	4.13
n-heptanol	1.3	2.8	1.15	2.8	1.15	6.6	4.08
CH_3NO_2	1.6	3.2	1.00	3.5	1.19	7.6	3.75

Figure 2.39: The Effect of Additives (1000 μg ml^{-1}) in the Organic Modifier on the Reverse Phase Partition Chromatography of a Standard Mixture on a Spherisorb ODS1 column. **Parameters:** see figure 2.36 except for: Flow Rate: 2 ml min^{-1} Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (70:30). Additives have been included in the MeOH (1000 μg ml^{-1}) as specified below



(a) none (b) n-Heptanol (c) Nitromethane (d) Pelargano Nitrile (e) n-Octanol

Figure 2.40: The Effect of Additives (1000 μg ml^{-1}) in the Organic Modifier on the Reverse Phase Partition Chromatography of a Standard Mixture on a μ-Bondapak ODS column

Parameters: see figure 2.27 except for: Flow Rate: 2.5 ml min^{-1}. Detection: UV at 260 nm. Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (80:20). Additives have been included in the MeOH as specified below.

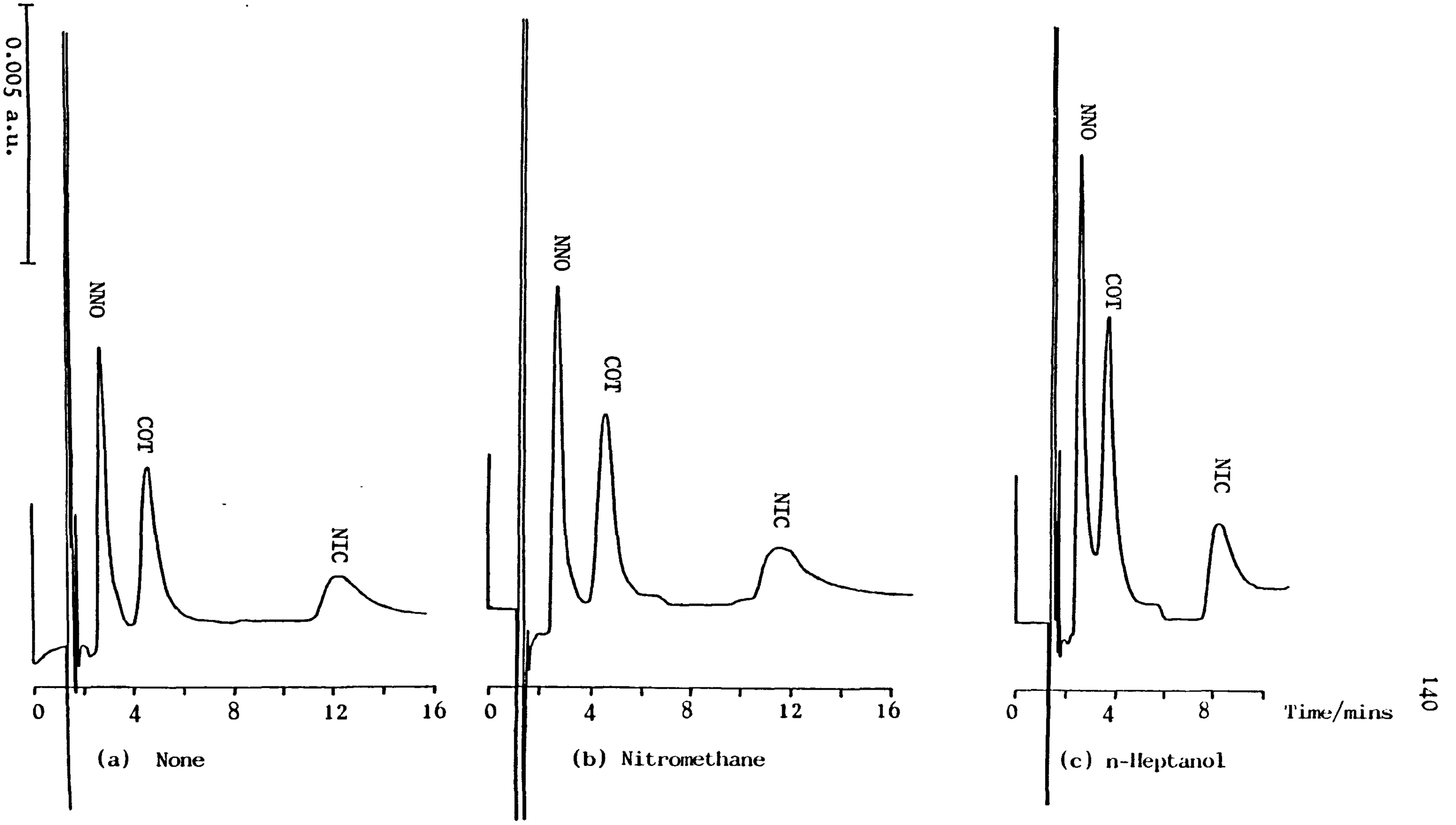
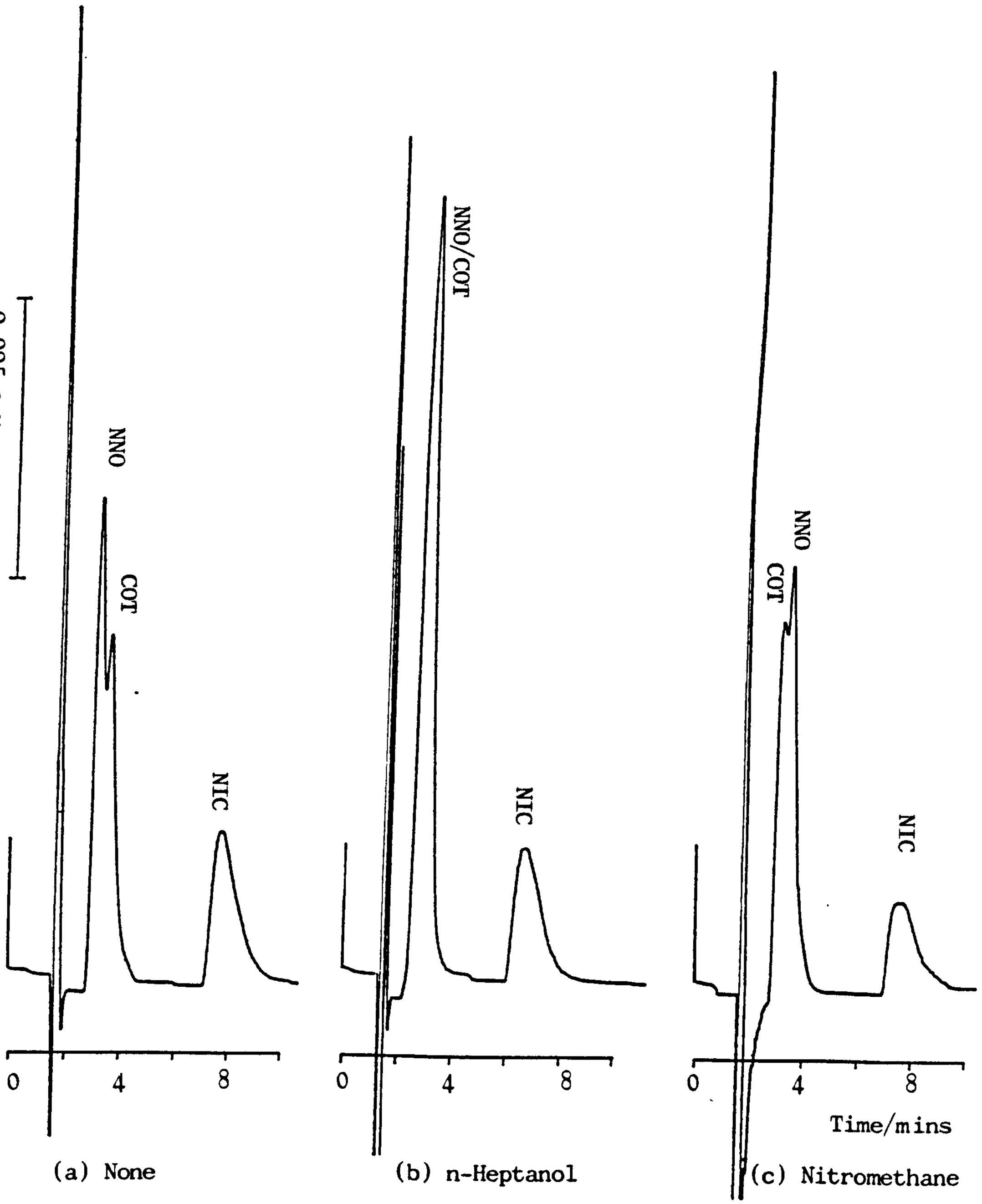


Figure 2.41: The Effect of Additives (1000 μg ml^{-1}) in the Organic Modifier on the Reverse Phase Partition Chromatography of a Standard Mixture on a μ-Bondapak ODS column

Parameters: see figure 2.40 except for:

Flow Rate: 2 ml min^{-1}.

Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (70:30). Additives have been included in the MeOH as specified below.



(a) None (b) n-Heptanol (c) Nitromethane

On a Partisil ODS2 column (25 cm x 4.6 mm ID), nicotine-1'-N-oxide is eluted after cotinine when the percentage of methanol in the eluent is greater than 30%. Nicotine is retained strongly by this ODS phase and requires a methanol content of 50% if the peak is to be visible above the baseline, see figure 2.42. Although the separation of nicotine from cotinine and nicotine-1'-N-oxide was easily achieved, cotinine and nicotine-1'-N-oxide were more difficult to resolve. Changes in the k' values of the standards vs. the percentage of MeOH in the mobile phase are illustrated in figure 2.43 and table 2.18.

3' Hydroxycotinine has been included in figure 2.43 and table 2.18, the inclusion of this nicotine metabolite will be discussed in section 2.11.

A Polymer Labs PL-RP-S 10 μ column (15 cm x 4.6 mm ID) was also examined. Using a mobile phase consisting of 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N : methanol (60:40), the k' values were markedly different. Under isocratic conditions a separation of all three standards was possible, as shown in figure 2.44, however nicotine-1'-N-oxide was virtually unretained by the column when the MeOH content of the eluent was as high as 40% but at the same time nicotine was very strongly retained by the PL-RP-S phase and was just visible above the baseline under the conditions employed.

As there were no pH restrictions on this resin based column, another mobile phase was made up as follows: 0.2% H_3PO_4, pH adjusted to 9.25 with Et_3N :methanol (60:40). At the higher pH value the retention times and k' values of all three components were shortened but in the case of the nicotine, still strongly retained, the peak remained broad and with a k' value of 25.4, see table 2.19 and figure 2.45.

Figure 2.42: Reverse Phase Partition Chromatography of a Standard Mixture on a Partisil ODS2 column

Parameters: Column: Partisil ODS2 (25 cm x 4.6 mm ID). Detection: UV at 260 nm.

Flow Rate: 1.0 ml min^{-1}. Sample: Standard mixture in H_2O (10 $\mu g\ ml^{-1}$). Sample Size: 20 µl.

Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (50:50).

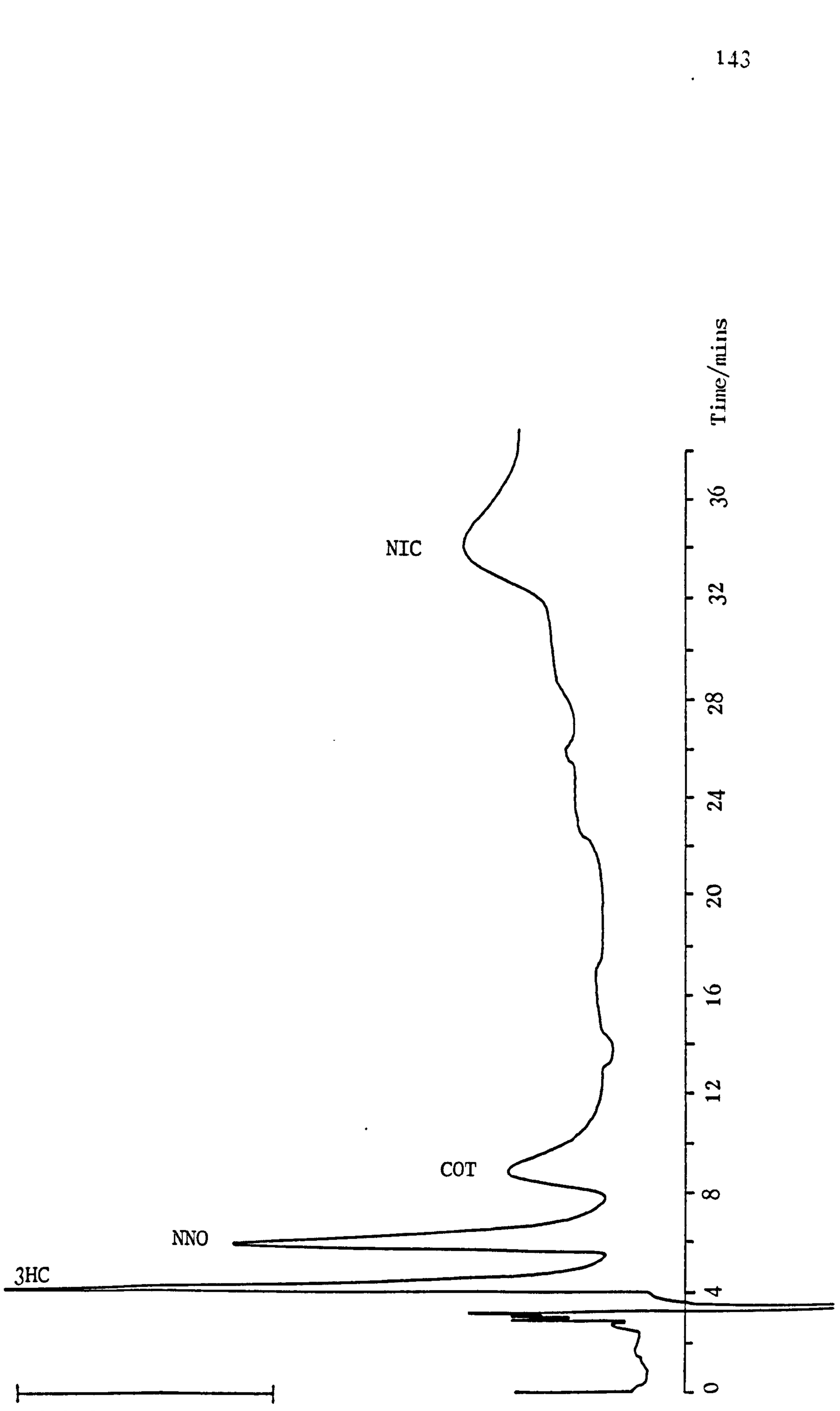


Figure 2.43: The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Partisil ODS2 column

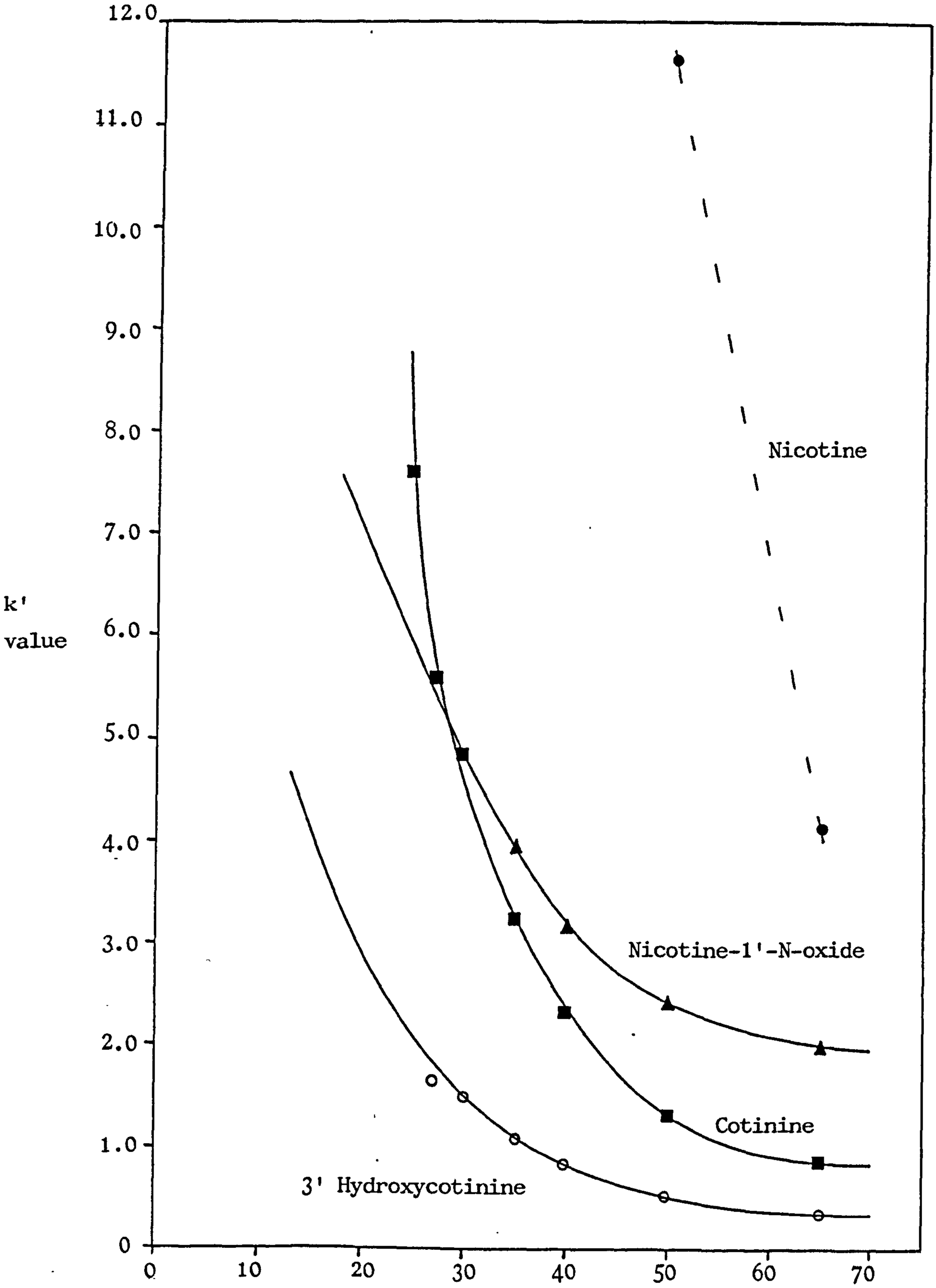


Table 2.18: The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Partisil ODS2 column

% Organic Modifier	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine		3' Hydroxy-cotinine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
25	2.8	-	-	24.2	7.64	-	-	8.6	2.07
27	2.8	-	-	18.6	5.64	-	-	7.4	1.64
30	2.8	16.5	4.89	16.6	4.93	-	-	7.0	1.50
35	2.8	13.9	3.96	11.9	3.25	-	-	6.0	1.14
40	2.8	11.6	3.14	9.1	2.25	-	-	5.2	0.86
50	2.8	9.6	2.43	6.4	1.29	35.5	11.68	4.1	0.46
65	2.4	7.1	1.96	4.5	0.88	12.3	4.12	3.2	0.33

Figure 2.44: Reverse Phase Partition Chromatography of a Standard Mixture on a PL-RP-S 10 μ, 100 Å column

Parameters: Column: PL-RP-S 10 μ (15 cm x 4.6 mm ID)
Flow Rate: 2.0 ml min^{-1}. Detection: UV at 260 nm.
Sample Size: 20 μl.
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (60:40)
Sample: Standard mixture in H_2O (10 μg ml^{-1})

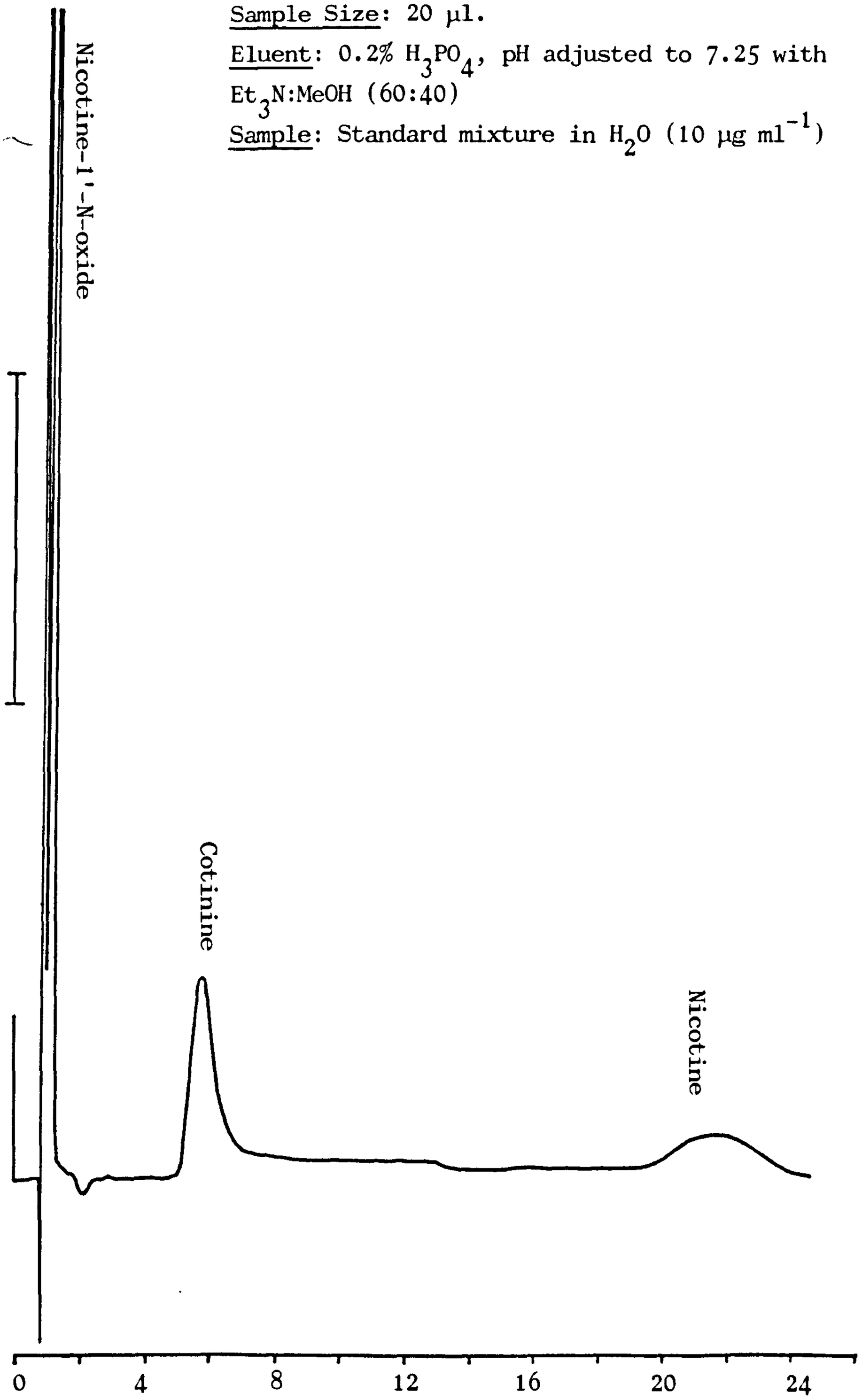
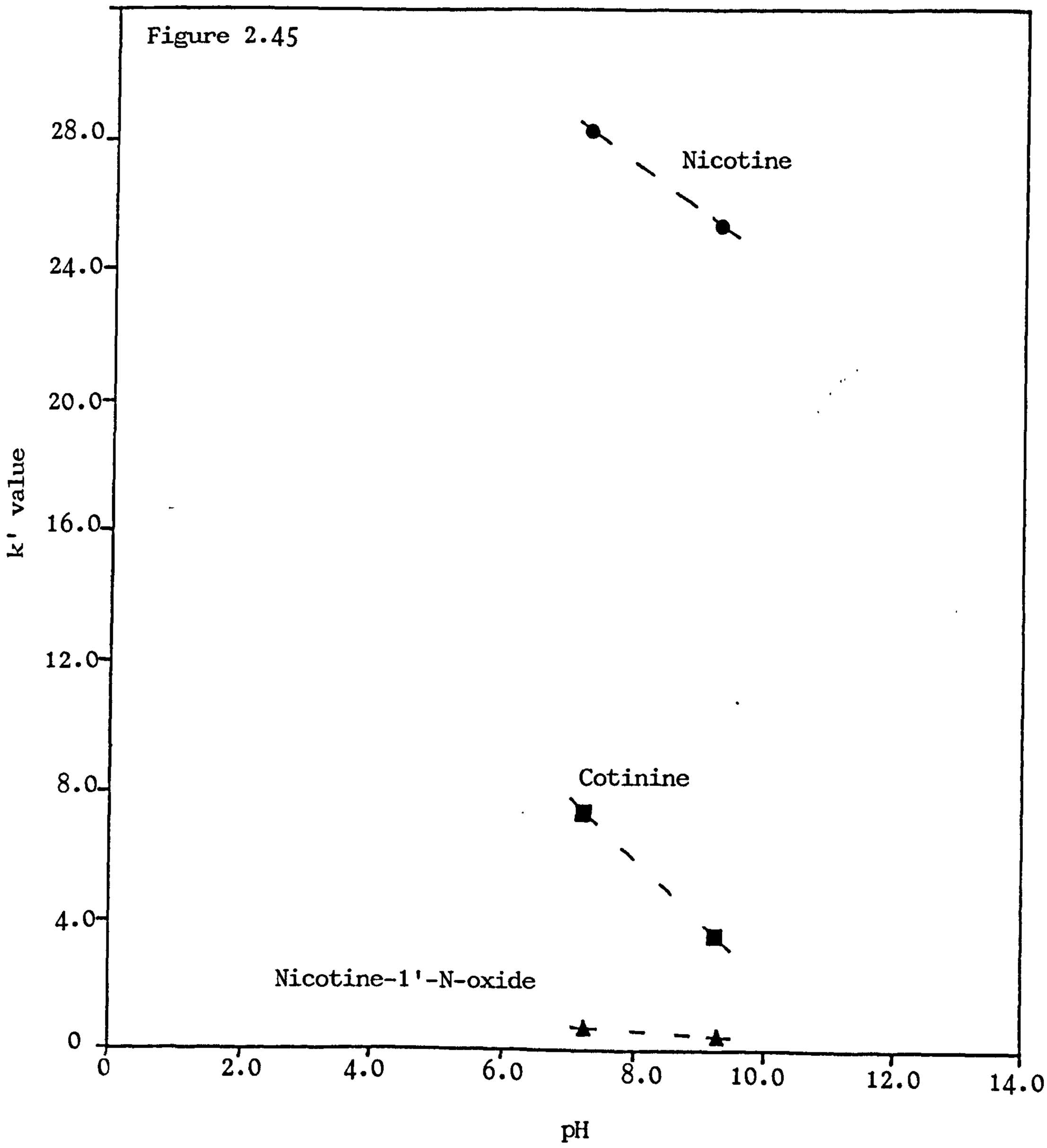


Table 2.19 and Figure 2.45:
The relationship between the retention time/capacity factor and pH of the mobile phase for standard components under reverse phase partition conditions on a PL-RP-S 10 μ 100Å column

Table 2.19

pH	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
7.25	1.5	2.6	0.73	12.4	7.26	44.0	28.33
9.25	1.4	2.0	0.43	6.3	3.5	37.0	25.40



On a Hypersil phenyl column (15 cm x 5 mm ID), the elution order was found to be different from that reported above for the ODS columns, with nicotine-1'-N-oxide being eluted first followed by nicotine and finally cotinine. Nicotine and cotinine were never completely resolved under any of the conditions employed; this is evident from figure 2.46 which shows the best separation of the three standards. The variation of k' with % MeOH is presented in table 2.20 and shown in graphical form in figure 2.47.

A Nucleosil NO_2 column (10 cm x 5 mm ID) gave the usual elution order: nicotine-1'-N-oxide, cotinine and nicotine. Unfortunately the nicotine-1'-N-oxide and cotinine peaks were not completely resolved regardless of the percentage of MeOH employed. The best separation between the three standards was achieved with a mobile phase containing only 10% MeOH, which is shown in figure 2.48 and which can be seen from the graph of k' value vs. % MeOH, figure 2.49 and table 2.21.

Several different packing materials have already been investigated; in order to choose the most suitable phase for further investigation, three criteria were used: (i) resolution, (ii) capacity factor of the first peak of interest and (iii) run time.
Baseline separation of all components of interest was sought, as the primary concern. For the sample analysis it was deemed necessary to have the first peak of interest with a k' value > 2, so as to avoid potential interference from early eluting species, unretained by the column and of no analytical interest. It was also considered desirable to keep the duration of the chromatographic analysis within reasonable limits (e.g. less than 30 minutes). The ultimate aim was to develop a method suitable for routine analysis of large numbers

Figure 2.46: Reverse Phase Partition Chromatography of a Standard Mixture on a Hypersil Phenyl column

Parameters: Column: Hypersil Phenyl (15 cm x 5 mm ID)
Flow Rate: 1.7 ml min^{-1}. Detection: UV at 260 nm.
Sample: Standard mixture in H_2O (10 µg ml^{-1}).
Sample Size: 20 µl.
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (80:20)

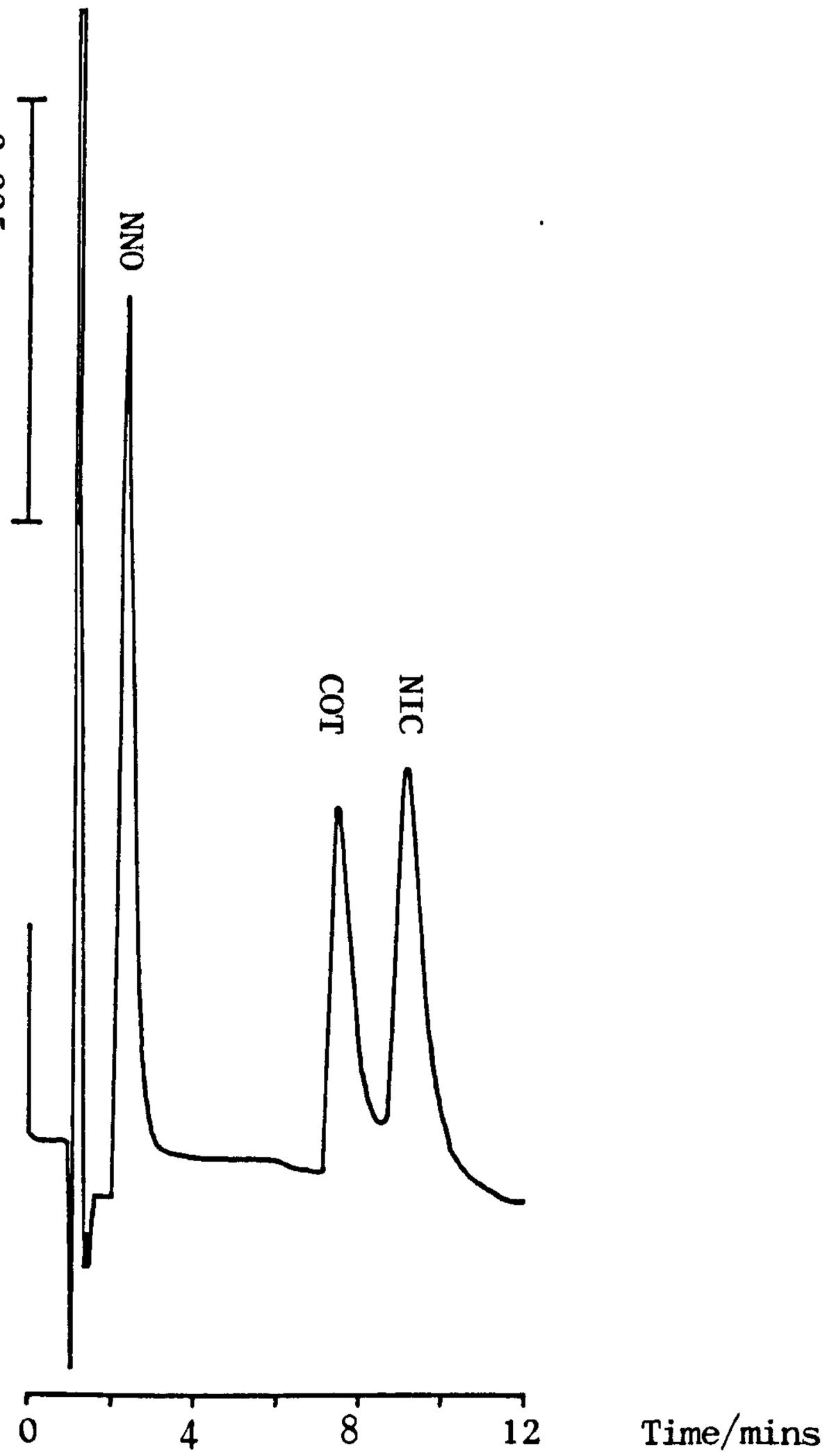


Table 2.20 and Figure 2.47:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Hypersil phenyl column

Table 2.20

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
20	2.0	4.3	1.15	16.1	7.05	13.4	5.70
30	2.0	3.5	0.75	8.7	3.35	7.5	2.75
40	1.9	2.8	0.47	5.3	1.79	5.3	1.79

Figure 2.47

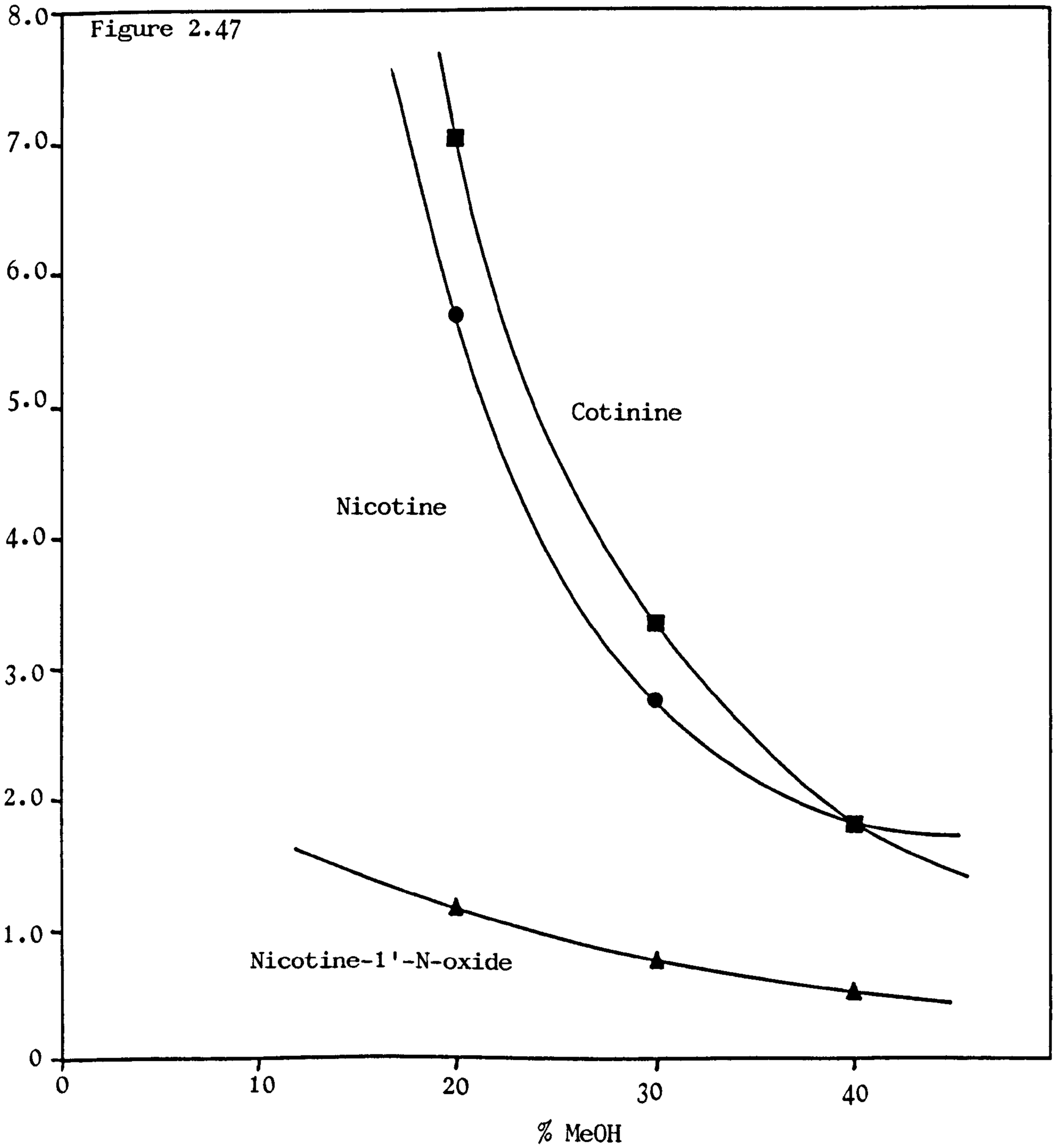


Figure 2.48: Reverse Phase Partition Chromatography of a Standard Mixture on a Nucleosil NO_2 column

Parameters: Column: Nucleosil NO_2 (10 cm x 5 mm ID)
Flow Rate: 0.5 ml min^{-1}. Detection: UV at 260 nm.
Sample: Standard mixture in H_2O (10 μg ml^{-1}).
Sample Size: 20 µl.
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH (90:10)

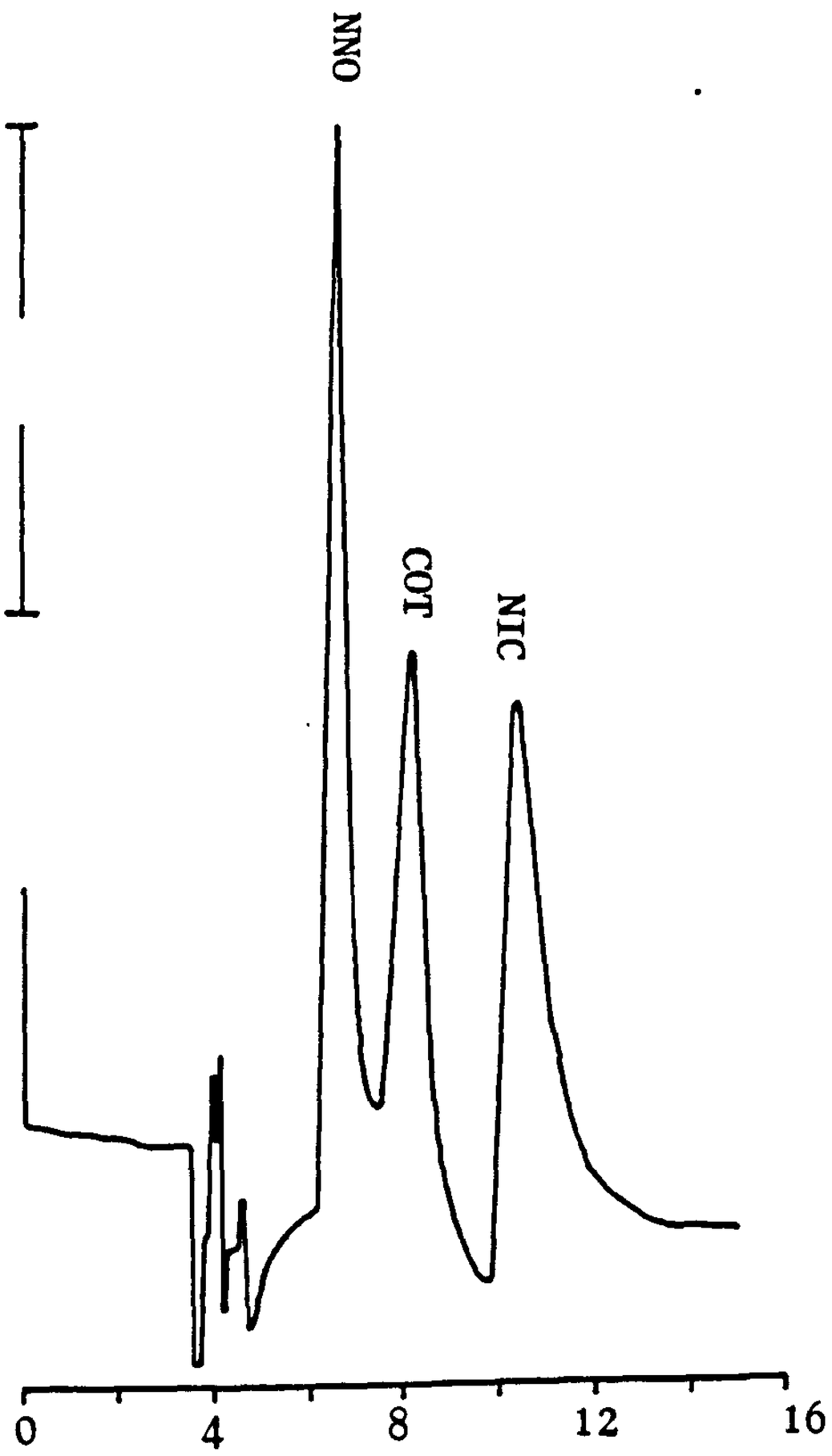


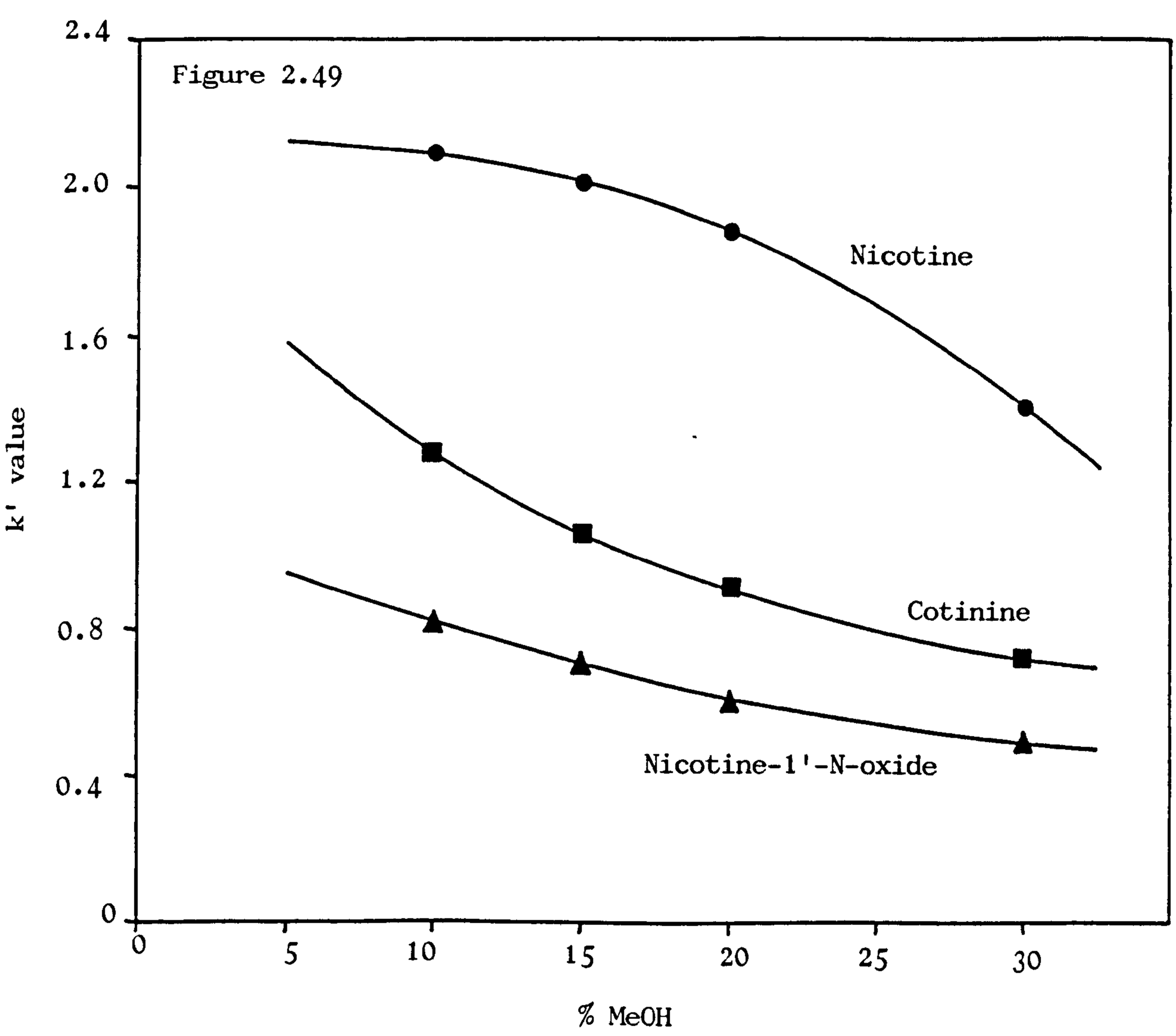
Table 2.21 and Figure 2.49:

The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Nucleosil NO_2 column

Table 2.21

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		Cotinine		Nicotine	
		t_R(mins)	k'	t_R(mins)	k'	t_R(mins)	k'
10	3.6	6.5	0.81	8.2	1.28	11.1	2.08
15	3.6	6.2	0.72	7.2	1.06	10.8	2.00
20	3.4	5.5	0.61	6.5	0.91	9.8	1.88
30	3.5	5.2	0.49	6.0	0.71	8.4	1.40

Figure 2.49



of biological samples, therefore it is important to emphasize these criteria initially.

Of the columns examined so far, μ-Bondapak gave a reasonable separation with the organic modifier at the 20% level in the mobile phase. Even with only 20% MeOH in the eluent, the k' value of nicotine-1'-N-oxide was still rather low at 1.31. Under the conditions employed it was not possible to achieve an increased value of k' for nicotine-1'-N-oxide and at the same time obtain a nicotine peak which was distinguishable from the baseline.

The R-Sil column, although resulting in similar separations to those obtained on the μ-Bondapak column, the best separation of the three standards did not give baseline resolution of cotinine and nicotine-1'-N-oxide.

Spherisorb ODS1, Spherisorb ODS2 and the Hypersil phenyl columns all showed nicotine-1'-N-oxide poorly retained, which was unfortunate as a very promising separation of the standards was evident in all these cases with the exception of the Hypersil phenyl column which showed a problem with the resolution of nicotine and cotinine.

The overriding problem on the Nucleosil column was undoubtedly the separation of NNO and cotinine, but k' values consistently less than 1 for nicotine-1'-N-oxide were also undesirable.

The PL-RP-S 10 μ column, although giving an excellent separation of the three standards, with very different k' values, was not considered for isocratic work for this same reason.

The Partisil ODS2 was also found to be unsuitable as the k' value for nicotine was very large unless the percentage of MeOH in the mobile phase exceeded 55%. When the percentage of MeOH was in

excess of 55%, nicotine-1'-N-oxide and cotinine did not result in a baseline separation and the k' value for cotinine was less than 1.3.

From the above trials, μ-Bondapak was the packing material considered most adaptable to the present problems, the main one being the low k' value of nicotine-1'-N-oxide. As an "improved" μ-Bondapak type packing material became available this was also included in the investigation. This packing material, Resolve C_{18} 5 μ, showed an increased retentiveness for all three standards. The relationship between k' values of the standards and the percentage of MeOH in the mobile phase is illustrated in table 2.22 and graphically in figure 2.50.

Although the Resolve C_{18} 5 μ packing material was unsuitable for isocratic work due to the incompatibility of the k' values which would result in a separation of all three standards, it was noted that nicotine-1'-N-oxide was more strongly retained and at the same time better separated from both cotinine and nicotine than on any other column investigated, see figure 2.51.

Anabasine, another alkaloid present in tobacco but not necessarily absorbed or produced in the body, was chromatographed. On the Resolve C_{18} 5 μ column it was possible to separate anabasine from the other standards, as shown in figure 2.52. The retention times and k' values were evaluated at different percentages of methanol and the k' values of both anabasine and nicotine vs. % methanol in the mobile phase are illustrated in table 2.23 and figure 2.53 respectively.

Anabasine was not included in further investigations. Nevertheless in the event of anabasine being present it is now known that in any subsequent analysis, it would not interfere with nicotine or

Table 2.22 and Figure 2.50:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Resolve C_{18} 5 μ column

Table 2.22

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide t_R (mins)	k'	Cotinine t_R (mins)	k'	Nicotine t_R (mins)	k'	N-ethyl nornicotine	
30	1.6	-	-	10.3	5.44	-	-	-	-
40	1.6	4.7	1.94	-	-	-	-	-	-
50	1.2	2.5	1.08	2.8	1.33	15.0	11.50	-	-
60	1.6	2.9	0.81	2.9	0.81	8.9	4.56	10.2	5.38
70	1.8	2.9	0.61	2.6	0.44	5.1	1.83	6.3	2.50

Figure 2.50

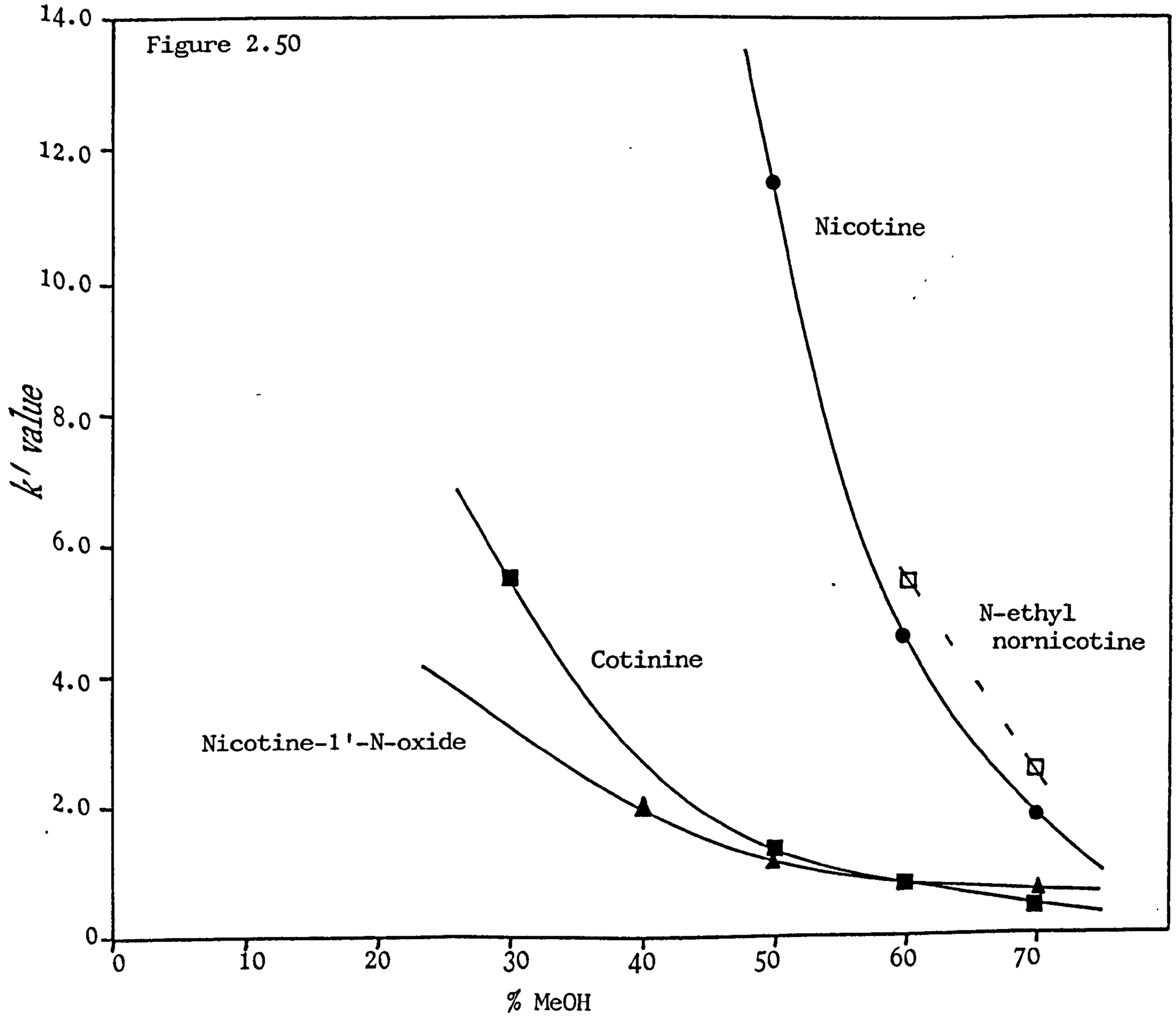
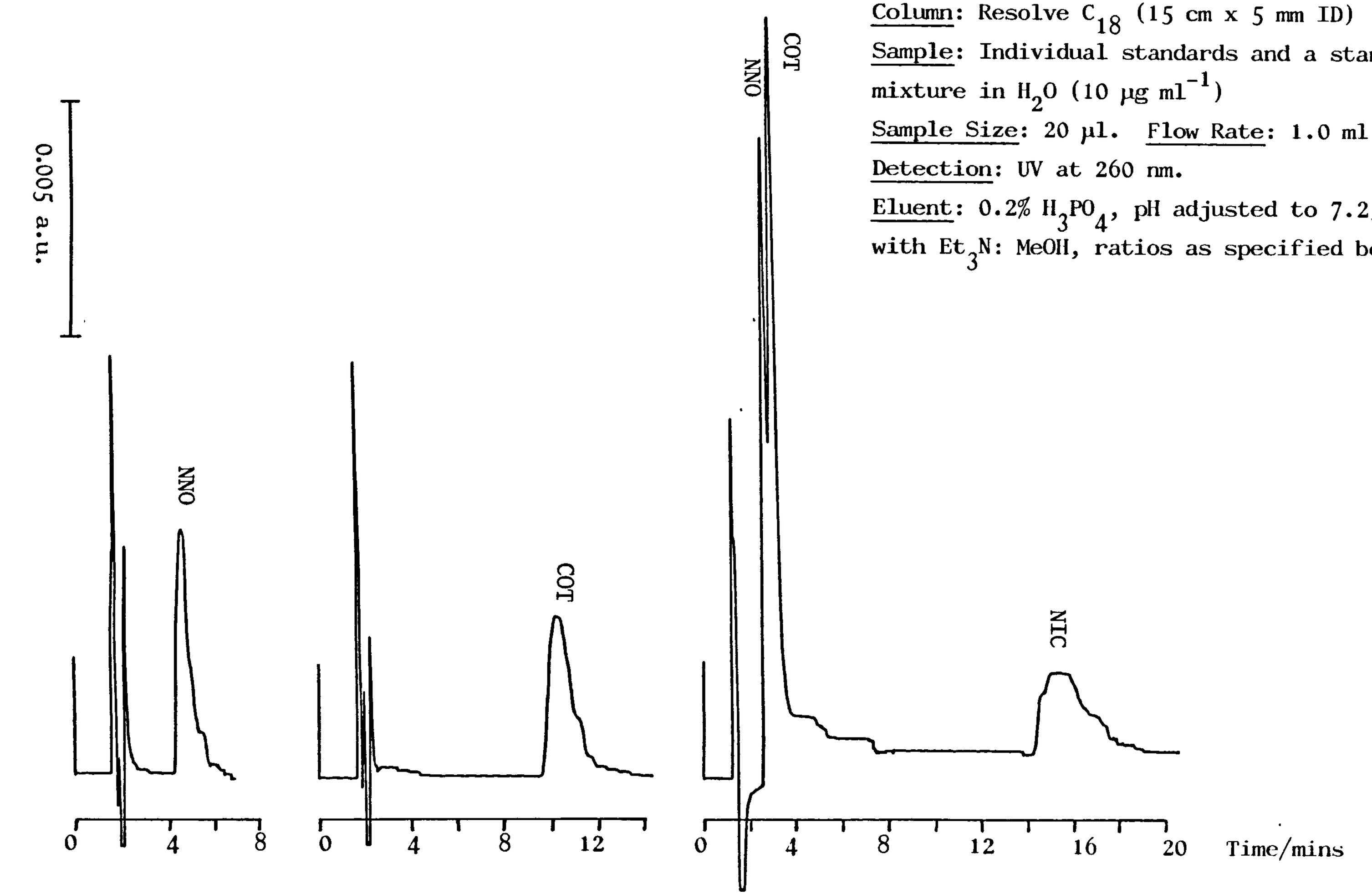


Figure 2.51: The Effect of Mobile Phase Composition (MeOH) content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a Resolve C_{18} 5 μ column

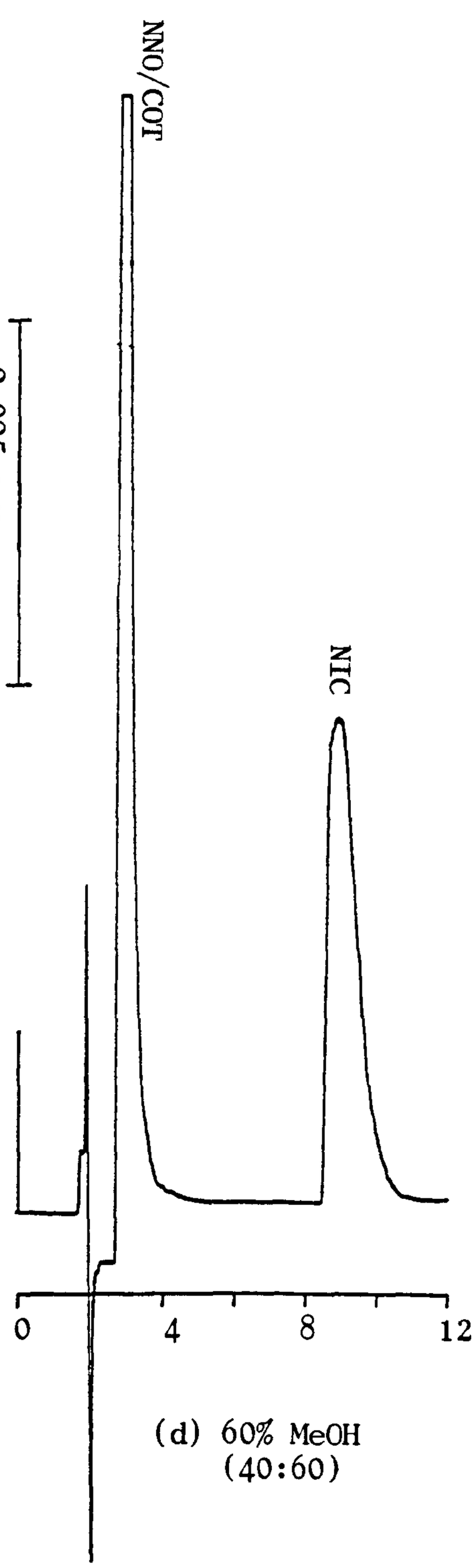
Parameters:

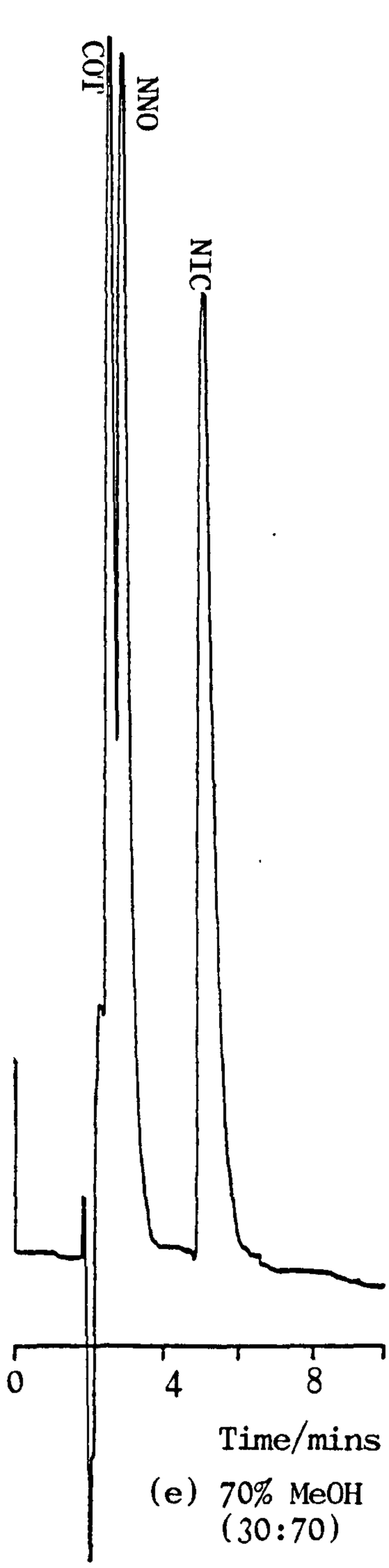
Column: Resolve C_{18} (15 cm x 5 mm ID)
Sample: Individual standards and a standard mixture in H_2O (10 μg ml^{-1})
Sample Size: 20 μl. Flow Rate: 1.0 ml min^{-1}
Detection: UV at 260 nm.
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N: MeOH, ratios as specified below.



(a) 40% MeOH (60:40) (b) 40% MeOH (60:40) (c) 50% MeOH (50:50)

Figure 2.51 (continued)





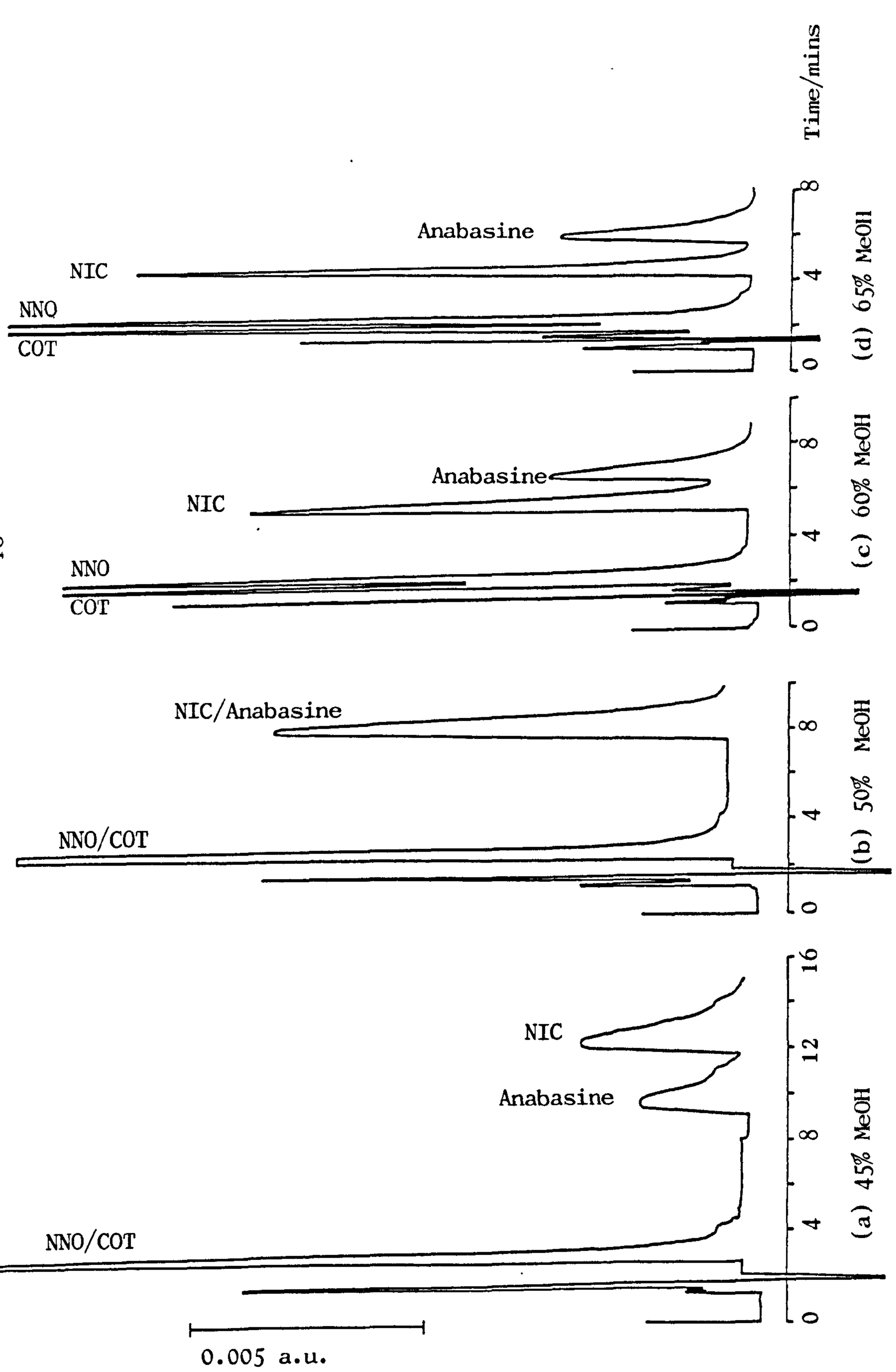


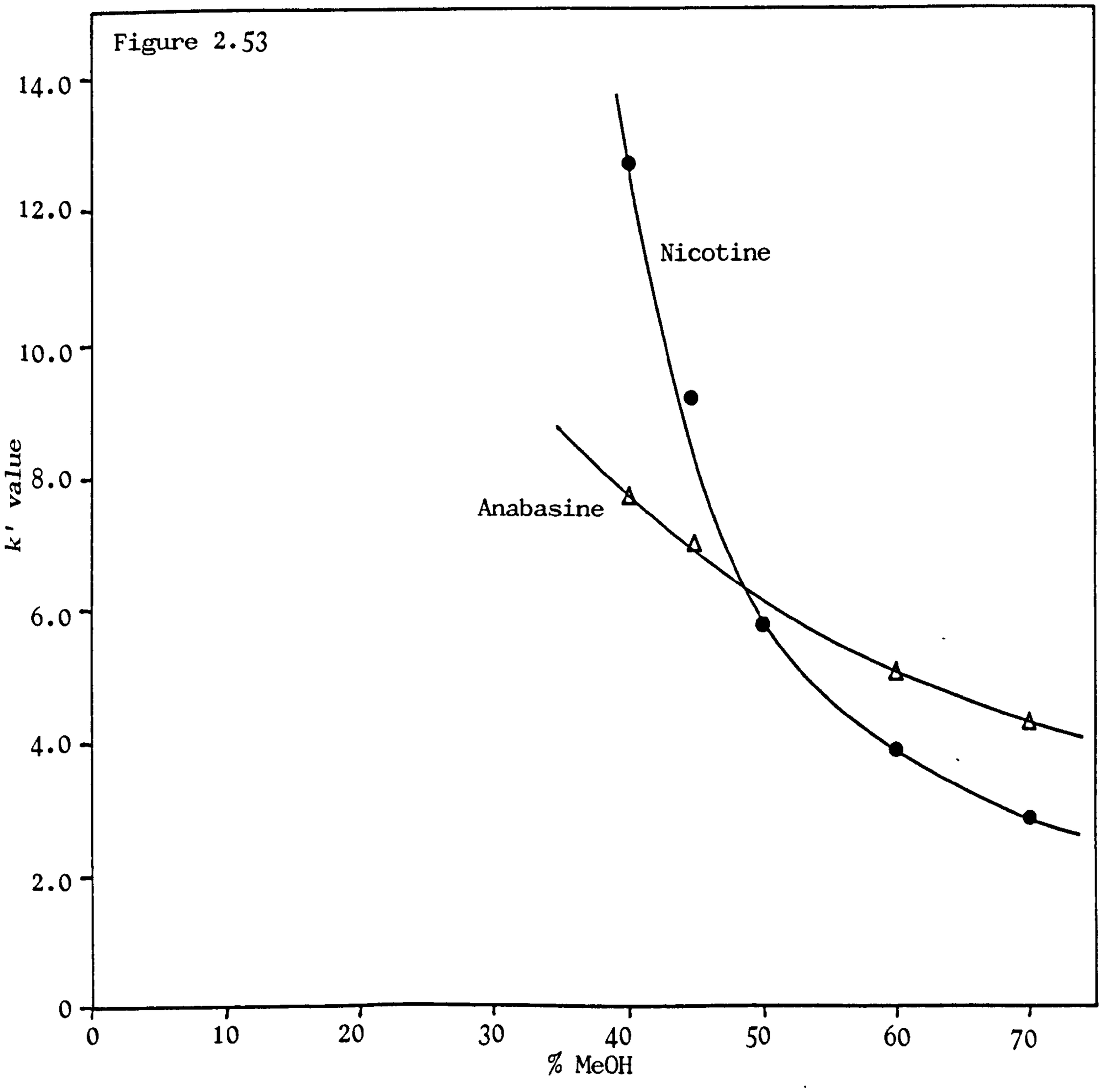
Figure 2.52: The Effect of Mobile Phase Composition (MeOH content) on the Reverse Phase Partition Chromatography of a Standard Mixture on a Resolve C_{18} 5 μ column. **Parameters:** see figure 2.51

Table 2.23 and Figure 2.53:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Resolve C_{18} 5 μ column

Table 2.23

% Organic Modifier (MeOH)	t_M (mins)	Nicotine		Anabasine	
		t_R(mins)	k'	t_R(mins)	k'
40	1.2	16.4	12.67	10.5	7.75
45	1.2	12.2	9.17	9.6	7.00
50	1.2	8.1	5.75	8.1	5.75
60	1.1	5.4	3.91	6.7	5.09
70	1.1	4.2	2.82	5.8	4.27

Figure 2.53



the metabolites included in this investigation.

Investigation of the Resolve C_{18} 5 μ column (15 cm x 5 mm ID), which showed the desired increase in the k' value of nicotine-1'-N-oxide in particular, but also the two other standards, led to the conclusion that, in order to achieve any further improvement in the chromatography, gradient elution would be necessary. Gradient elution would enable the standards to be eluted in a reasonable time and with a peak shape suitable for quantitation.

2.9.3 Gradient Elution

A high pressure gradient elution system was set up to replace the isocratic one in use. Initial problems with the use of the gradient system were encountered. The most serious problem occurred on running a blank gradient, that is one where no injection of standards or sample was made, where fluctuations in the baseline were observed. A classical problem in absorbance detection is distinguishing between genuine absorbance and a change in energy reaching the detector because of refractive index (RI) changes in the sample cell. Refractive index effects are significant in gradient elution work and especially when high sensitivity is required due, for example, to low sample concentration. The UV detector had been operating at the sensitivity setting of 0.02 a.u.f.s. into 10 mV f.s.d. To overcome the need for such a high sensitivity setting the 20 μl loop injector already in use was replaced by a 50 μl loop and the sensitivity lowered to 0.04 a.u.f.s.

An injection of 50 μl onto a conventional analytical column, with a 5 mm internal diameter, may cause column overloading; however

fronting of the peaks, which is an indication of overloading, was not evident and for the initial gradient trials, this danger was overlooked.

Chromatograms showing nicotine, cotinine, anabasine and nicotine-1'-N-oxide standards using the Resolve C_{18} 5 μ column and gradient elution are shown in figure 2.54. The peak shape of nicotine, in particular, has shown a dramatic improvement.

It was reported in the operating manual for the Waters solvent programmer[197] being used that, whenever possible, it is desirable to premix solvents at the initial and final concentration levels, that is to premix those end-point concentrations as the pump 'A' and pump 'B' solvents respectively. Solvent premixing was considered to increase resolution and improve operational performance as the programmer could then run from 0%B to 100%B. It was found that pre-mixing the solvents to correspond with the end-points of a suitable programme did not make a significant difference in general. Although satisfactory chromatograms were obtained, premixing added an unnecessary complication to the method and was therefore not considered further.

Having established that the Resolve C_{18} 5 μ packing material offered certain advantages over the μ-Bondapak material, especially as gradient elution was now available, the possibility of using Resolve C_{18} 5 μ material in the Waters flexible-walled columns was looked on as a further opportunity for improvement. The flexible-walled columns, already discussed in Section 2.5.4, have an 8 mm internal diameter and so the use of a 100 μl injector loop was also incorporated. The separation obtained was excellent, as shown by figure 2.55.

The gradient programmer in use offered only a single gradient

Figure 2.54: Reverse Phase Partition Chromatography of Standard Mixtures on a Resolve C_{18} 5 μ column using Gradient Elution.

Parameters: see figure 2.51 except for:

Flow Rate: 1.1 ml min^{-1}. Sample Size: 50 μl.

Eluent: Liquid A: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N, Liquid B: MeOH. Gradient programme #5, 32%→ 55% B over 10 mins.

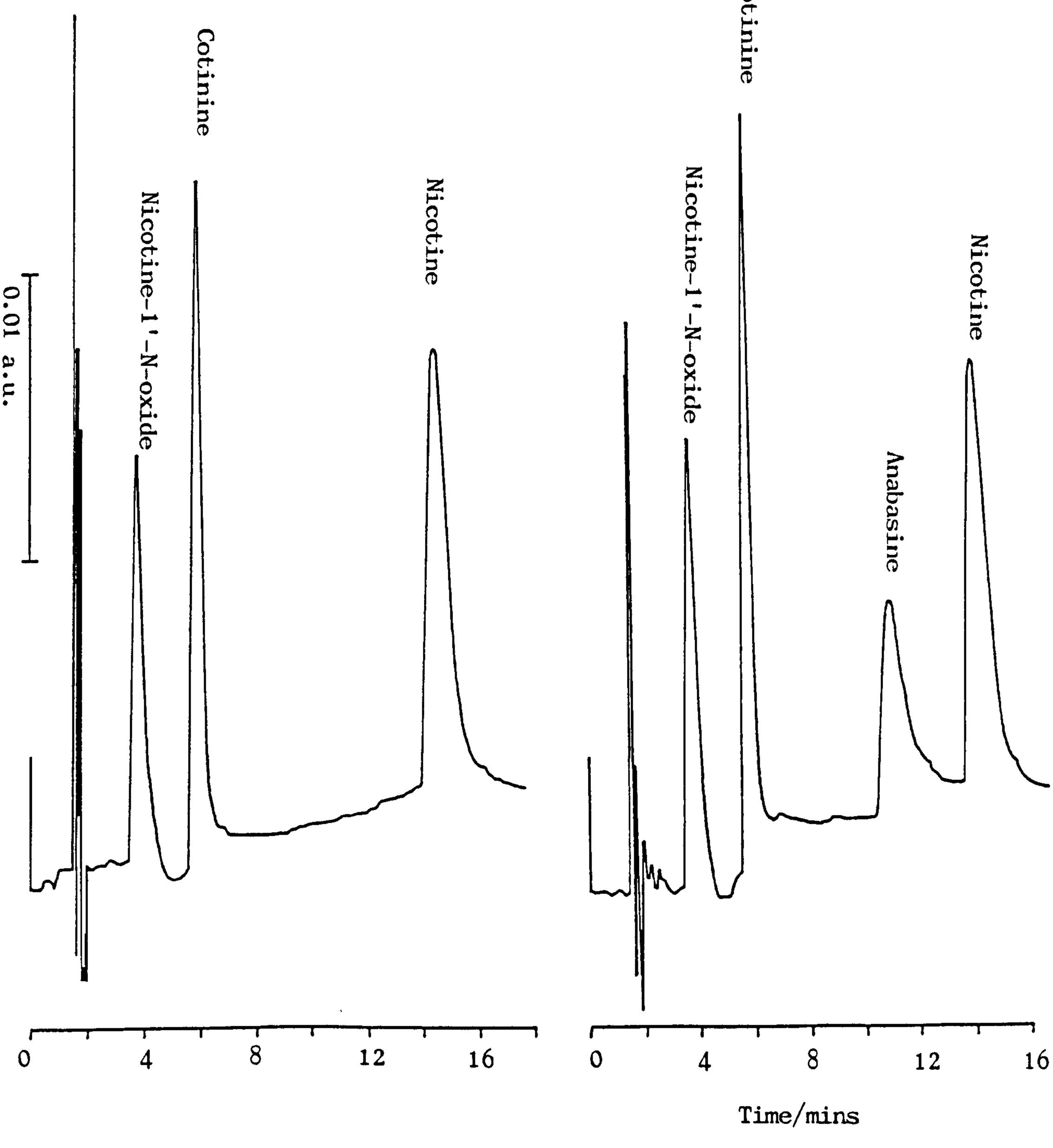
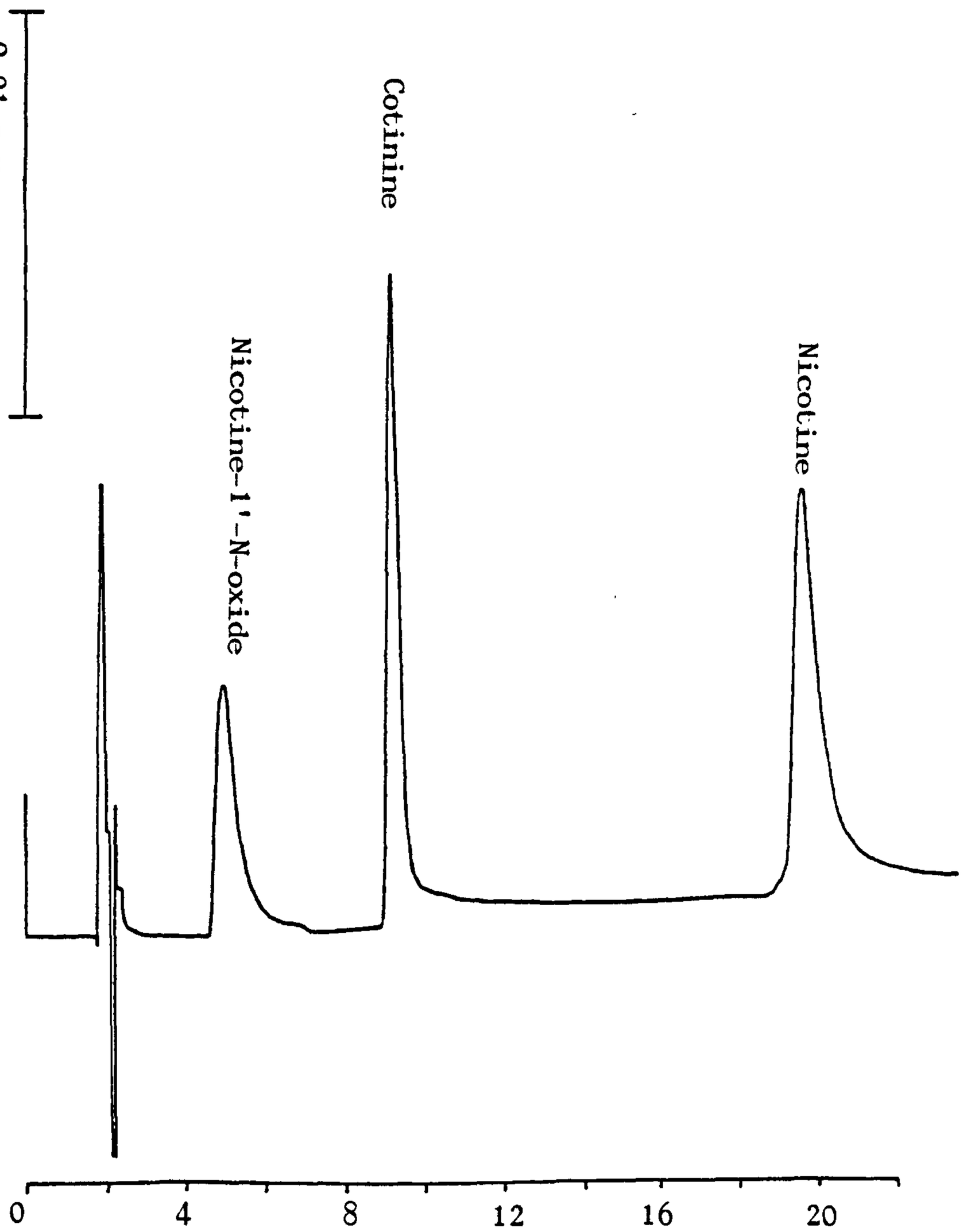
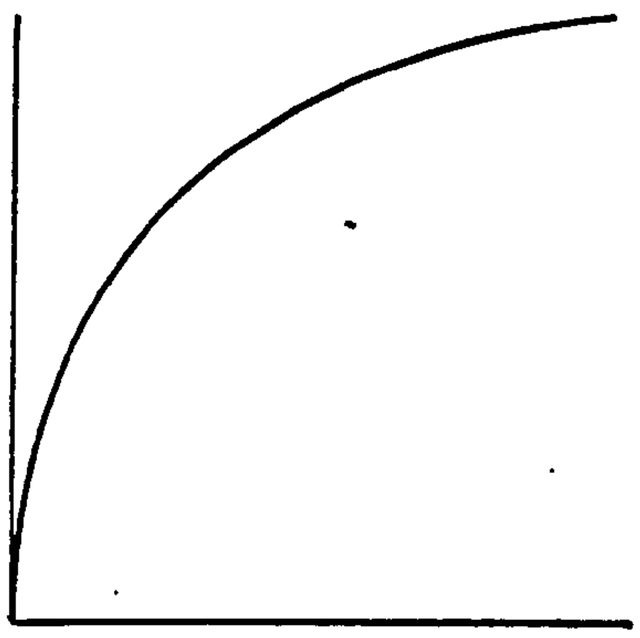


Figure 2.55: Reverse Phase Partition Chromatography of a Standard Mixture on a Resolve C_{18} 5 μ Radial PAK cartridge in the RCM-100 using Gradient Elution.

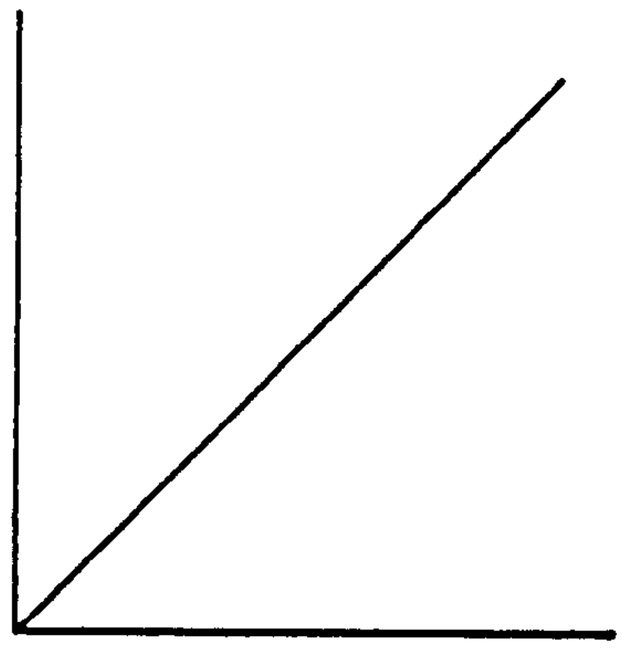
Parameters: Column: Radial PAK Resolve C_{18} 5 μ cartridge (10 cm x 8 mm ID) in a Waters Radial Compression Unit RCM-100.
Detection: UV at 260 nm.
Sample: Standard mixture in H_2O (10 μg ml^{-1})
Sample Size: 100 μl. Flow Rate: 1.5 ml min^{-1}
Eluent: Liquid A: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N, Liquid B: MeOH. Gradient programme # 6 25% → 58% B, over 14 mins.



profile per run. The two programmes used were numbers 5 and 6 whose profiles are shown below.



prog # 5



prog # 6

On close examination of figure 2.55 it may have been desirable to alter the gradient profile after cotinine had been eluted, therefore speeding up the total analysis time and at the same time improving the chromatography of nicotine still further.

2.10 The Search for an Internal Standard

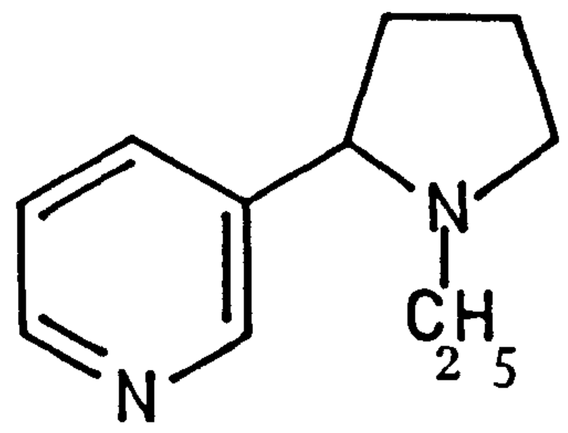
At this point a successful separation of nicotine and two of its metabolites had been achieved. Before proceeding to quantitative analysis it was considered essential to find a suitable internal or chromatographic standard.

An internal standard is a component added to the sample or mixture of standards before extraction. The internal standard has to be completely resolved chromatographically from the other components of interest, is not already present in the samples to be analyzed,

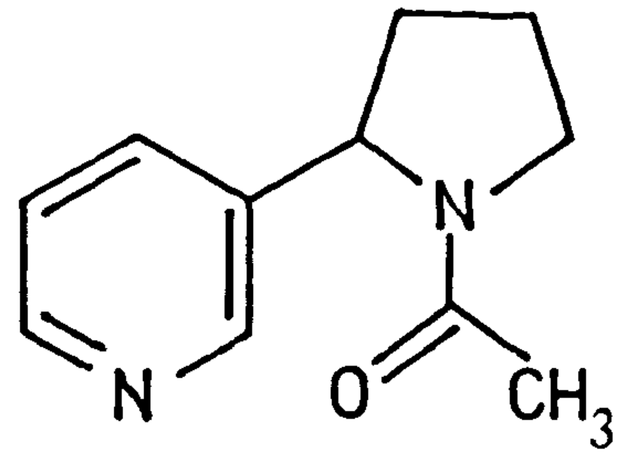
and does not have any interference effects. Ideally it should be readily available and inexpensive to buy. It is often found that the internal standard is similar in structure to the component(s) of interest making it more likely that it can be taken through the extraction procedure, however similarity of structure is not a pre-requisite. If it is not possible to find a suitable internal standard then a chromatographic standard can be used. All the criteria listed above apply, except that a chromatographic standard is not taken through the extraction procedure. The internal or chromatographic standard permits the operating conditions to vary from sample to sample, but the internal standard is the most desirable as it also monitors the efficiency of the extraction procedure.

Initially, the objective was to find one or more compounds which were soluble in the mobile phase and could be completely resolved from all other components of interest with little or no modification of the gradient profile already in operation.

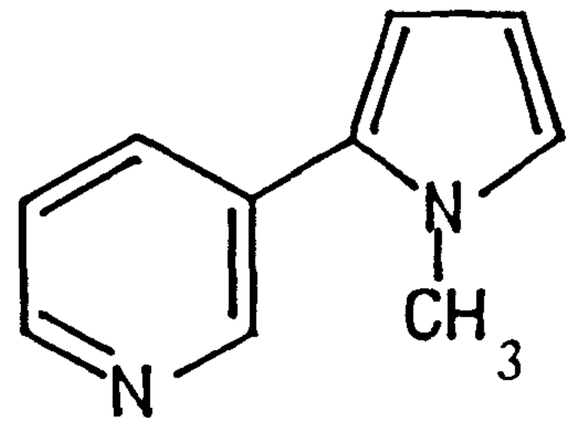
N-ethyl nornicotine was the first compound considered as an internal standard. Its structure is shown in figure 2.56. As expected, nicotine and N-ethyl nornicotine were a most difficult pair to separate. Having chosen the Resolve C_{18} 5 μ column (20 cm x 5 mm ID) as the separation column, using the gradient programmer achieved a separation of the three standards in a reasonable time. N-ethyl nornicotine was added to the mixture of standards and although variations in the gradient programme already in use were investigated, nicotine and N-ethyl nornicotine were eluted on every occasion with overlapping peaks as shown in figure 2.57. It was concluded that N-ethyl nornicotine was an unsuitable internal standard as it could not be



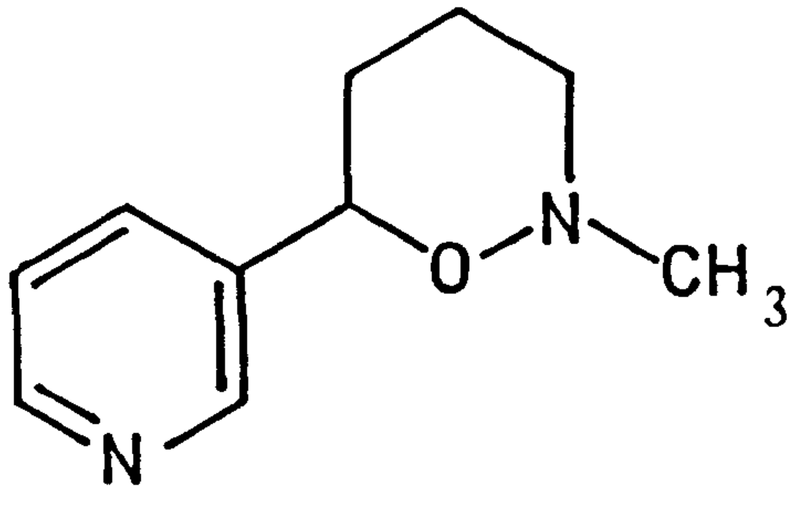
N'-ethyl nornicotine



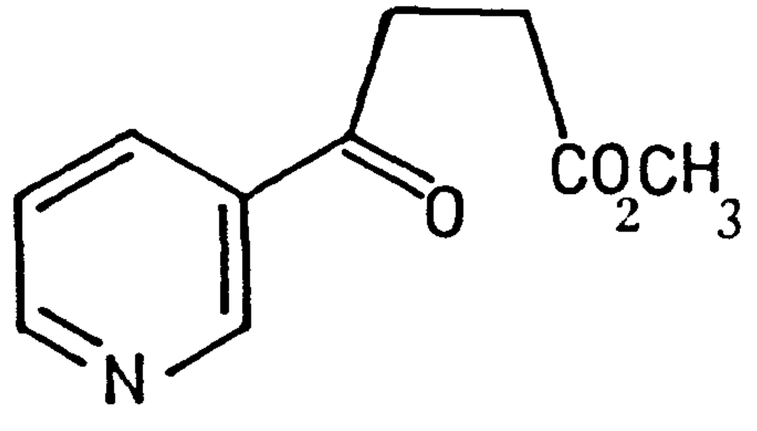
N'-acetyl-S-(-)-nornicotine



β-Nicotyrine



2 methyl-6-(3-pyridyl)-tetra-hydro-(1,2)-oxazine



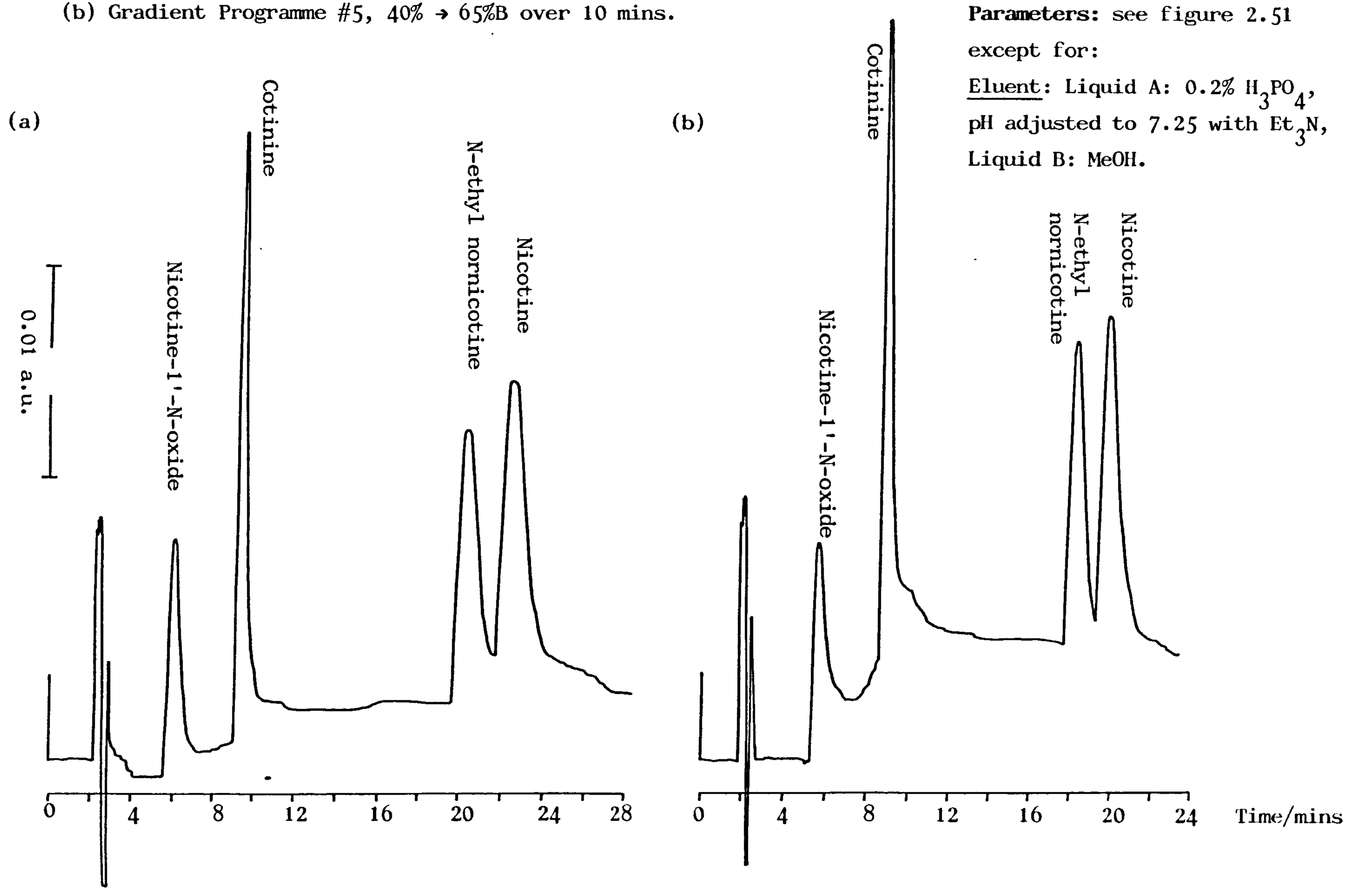
Methyl-4-(3-pyridyl)-4-oxo-butyrate

Figure 2.56: Internal standard structures

Figure 2.57: Reverse Phase Partition Chromatography of a Standard Mixture including N-ethyl nornicotine on a Resolve C_{18} 5 μ column using Gradient Elution. (a) Gradient Programme #5, 40% → 60%B over 10 mins; (b) Gradient Programme #5, 40% → 65%B over 10 mins.

Parameters: see figure 2.51 except for:

Eluent: Liquid A: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N, Liquid B: MeOH.



resolved 100% from all other components.

One interesting result from the chromatography of N-ethyl nornicotine was its behaviour on the Nucleosil NO_2 column. Although the three standards, nicotine-1'-N-oxide, cotinine and nicotine, were not resolved successfully on this column, N-ethyl nornicotine and nicotine were separated when the mobile phase contained 20% MeOH, as shown in figure 2.58. The percentage of methanol was decreased in an attempt to separate the components of interest, nicotine-1'-N-oxide, cotinine and nicotine. However, with only 10% MeOH in the mobile phase, it was noted that cotinine and N-ethyl nornicotine co-eluted.

Several compounds were selected at random; however, most were found to be unsuitable for one of several reasons. The following compounds were not soluble enough in the mobile phase:

(i) diphenyl methyl carbinol

(ii) N,N-dimethylaniline

(iii) 1,4 dichlorobenzene

(iv) diphenyl amine

(v) p-nitrobenzaldehyde

(vi) medazepam

(vii) nitrazepam .

(viii) 8 hydroxyquinoline, and

(xi) o-nitrophenol

both gave a pale yellow coloured solution on standing.

Failing success with compounds chosen at random, four compounds were synthesized by our industrial sponsors, to be tested as possible internal or chromatographic standards. The four compounds were:

Figure 2.58: The Effect of Mobile Phase Composition (MeOH content) on the Reverse Phase Partition Chromatography of Standard Mixtures (one including N-ethylnornicotine) on a Nucleosil NO_2 column.
Parameters: see figure 2.48 except for:
Eluent: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N:MeOH.
Ratios as specified below.

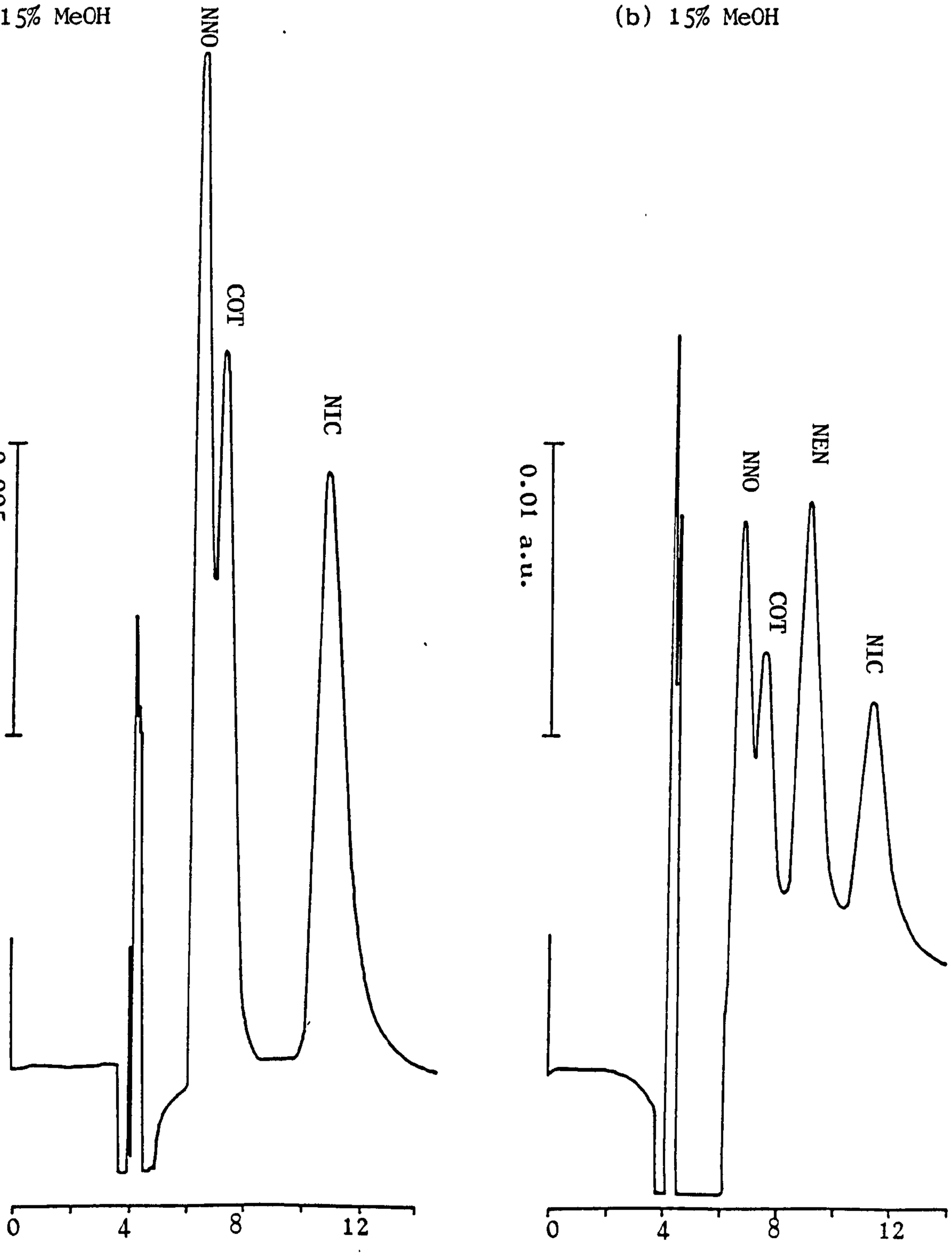


Figure 2.58 (continued)

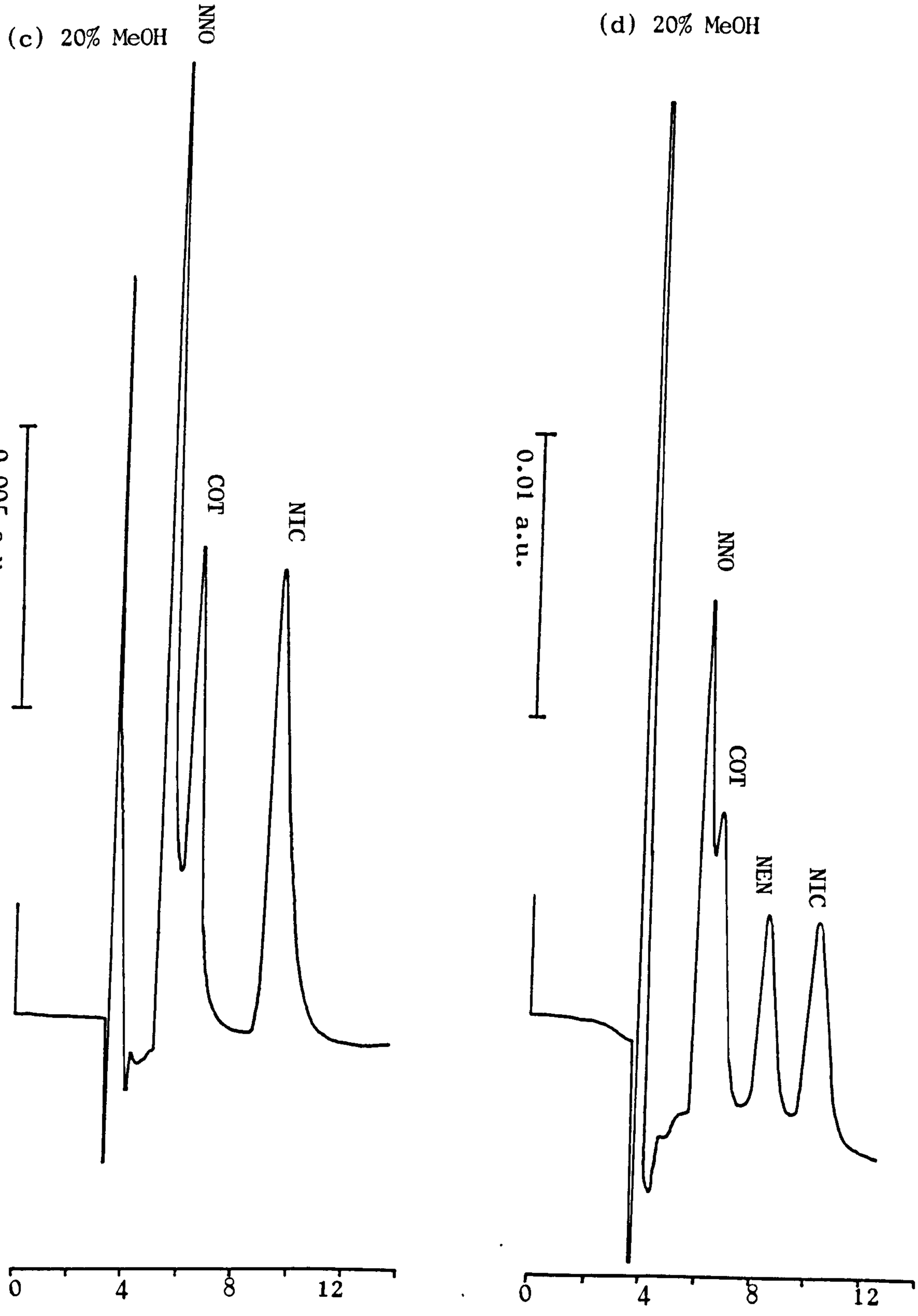
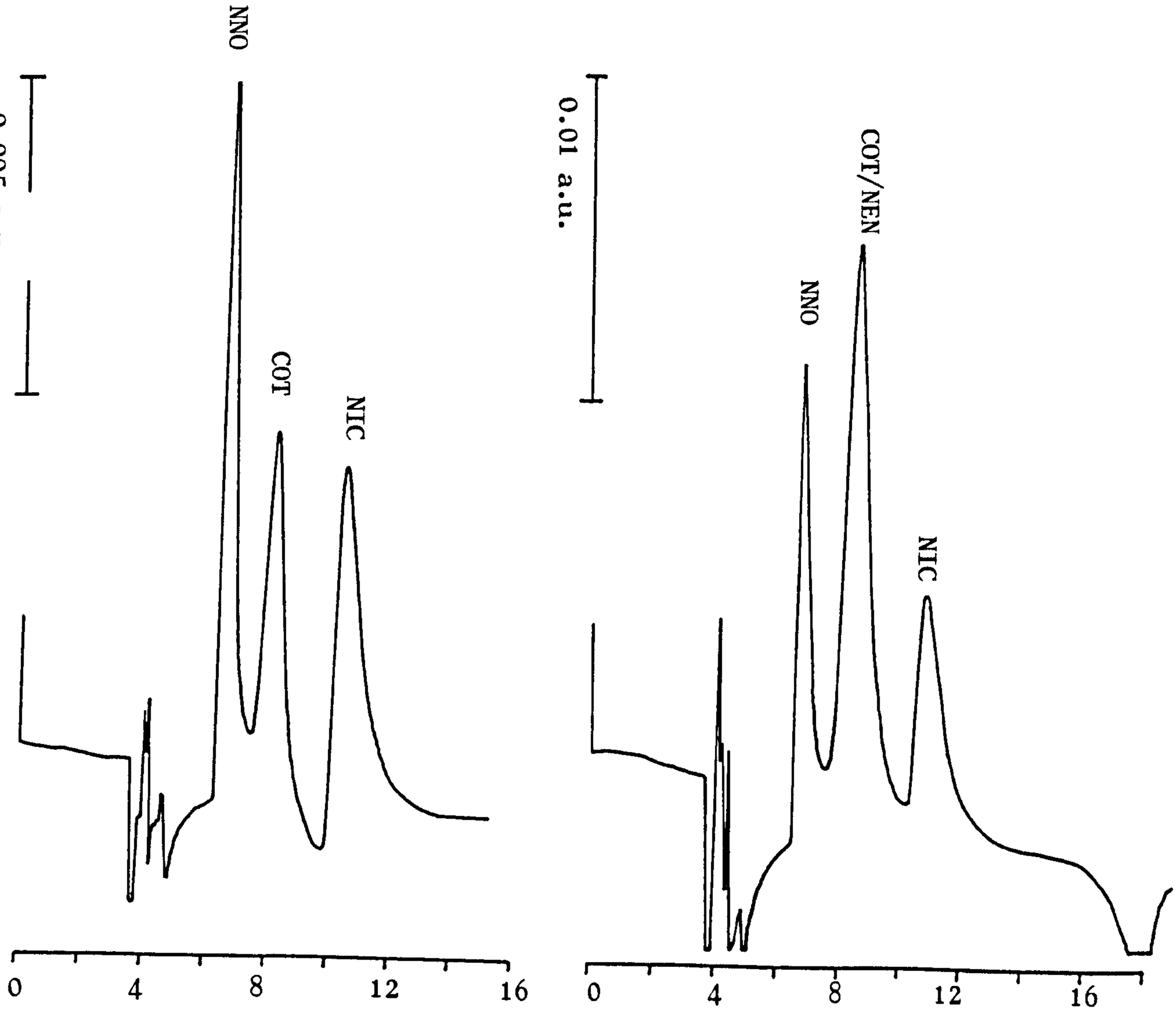


Figure 2.58 (continued)

(e) 10% MeOH

(f) 10% MeOH



(a) methyl-4-(3-pyridyl)-4-oxo-butyrate

(b) 2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine

(c) β-Nicotyrine and

(d) N'-Acetyl-S-(-)-nornicotine

and their structures are also shown in figure 2.56.

Using the following programmed run: prog #5, 3 min initial hold at 23% MeOH, 23% → 58% MeOH over 14 mins, the retention times and k' values of all four compounds were noted and compared with those of the components of interest, nicotine, cotinine and nicotine-1'-N-oxide.

All four compounds gave satisfactory retention times and k' values, as shown in table 2.24; however, methyl-4-(3-pyridyl)-4-oxo-butyrate gave two peaks. The peak height ratios observed for methyl-4-(3-pyridyl)-4-oxobutyrate changed on repeating the injection of a 10 $\mu g\ ml^{-1}$ solution in water over several days, see figure 2.59. Methyl-4-(3-pyridyl)-4-oxobutyrate was discarded as a possible internal standard due to its unstable nature in water.

Chromatograms of the other three compounds are shown in figures 2.60 and 2.61.

It was noted that on storage β-nicotyrine solution 1000 $\mu g\ ml^{-1}$ in water was found to change from a colourless solution to one with a yellow tint, over a period of weeks, even though it was stored in the refrigerator at 4°C when not in use. For this reason it was also discarded.

Table 2.24: Retention times and capacity factors for standard components and potential internal standards on a Resolve C_{18} 5 μ Radial PAK cartridge in a Waters RCM-100 unit using gradient HPLC

Component	t_R(mins)	k' * value
Nicotine-1'-N-oxide	6.67	1.56
Cotinine	12.04	3.63
N'-acetyl nornicotine	13.48	4.18
Methyl-4-(3-pyridyl)-4-oxo-butyrate	15.27 (small peak at) (5.70)	4.87 (1.19)
2-methyl-6-(3-pyridyl)-tetra-hydro-(1,2)-oxazine	21.32	7.20
Nicotine	23.26	7.95
β-Nicotyrine	25.55	8.83

* t_M = 2.6 mins

2.11 The Inclusion of 3' Hydroxycotinine

When 3' hydroxycotinine, which had only very recently been identified and synthesized, became available it was hoped that the method developed was flexible enough to cope with the addition of this metabolite. The objective was to fit 3' hydroxycotinine into the gradient run already in use, or a slightly modified one, resolved from all other components of interest.

3' Hydroxycotinine was eluted after nicotine-1'-N-oxide on the Resolve C_{18} 5 μ cartridge; however, a lower percentage of MeOH was

Figure 2.59: Reverse Phase Partition Chromatography of Methyl-4-(3-pyridyl)-4-oxo-butyrate over a one-week period. **Parameters:** see figure 2.55, except for: Flow Rate: 1.1 ml min^{-1}
Sample: Standard solution in H_2O (10 µg ml^{-1})
Eluent: 3 min initial hold at 23% B. Gradient prog. #5, 23% → 58%B, over 14 mins

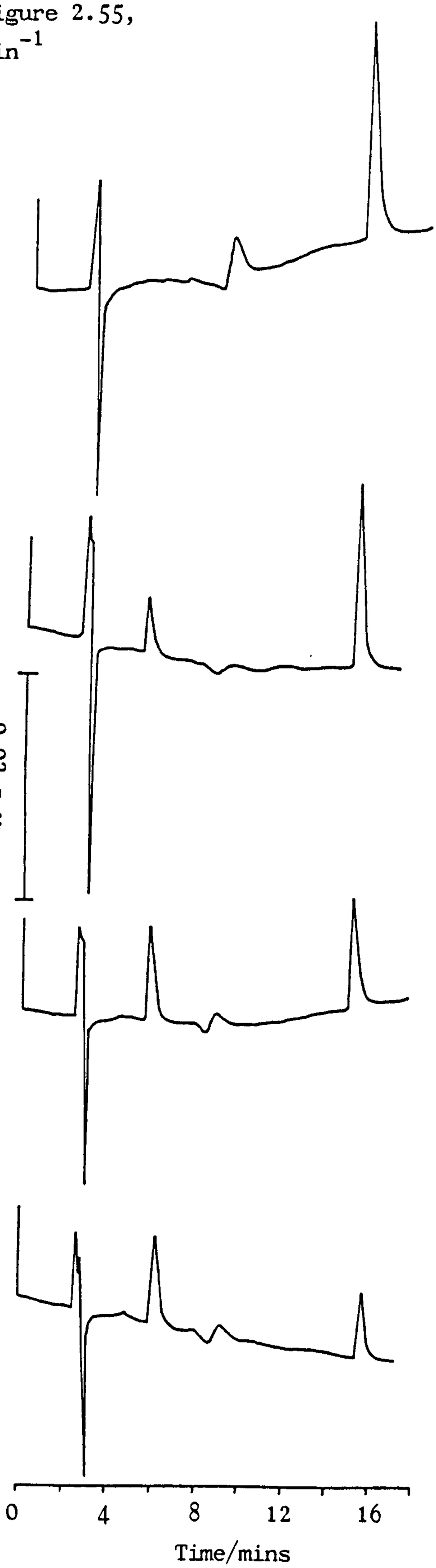


Figure 2.60: Reverse Phase Partition Chromatography of Three Possible Internal Standards on a Resolve C_{18} 5 μ Radial PAK Cartridge in the RCM-100 using Gradient Elution

Parameters: see figures 2.59

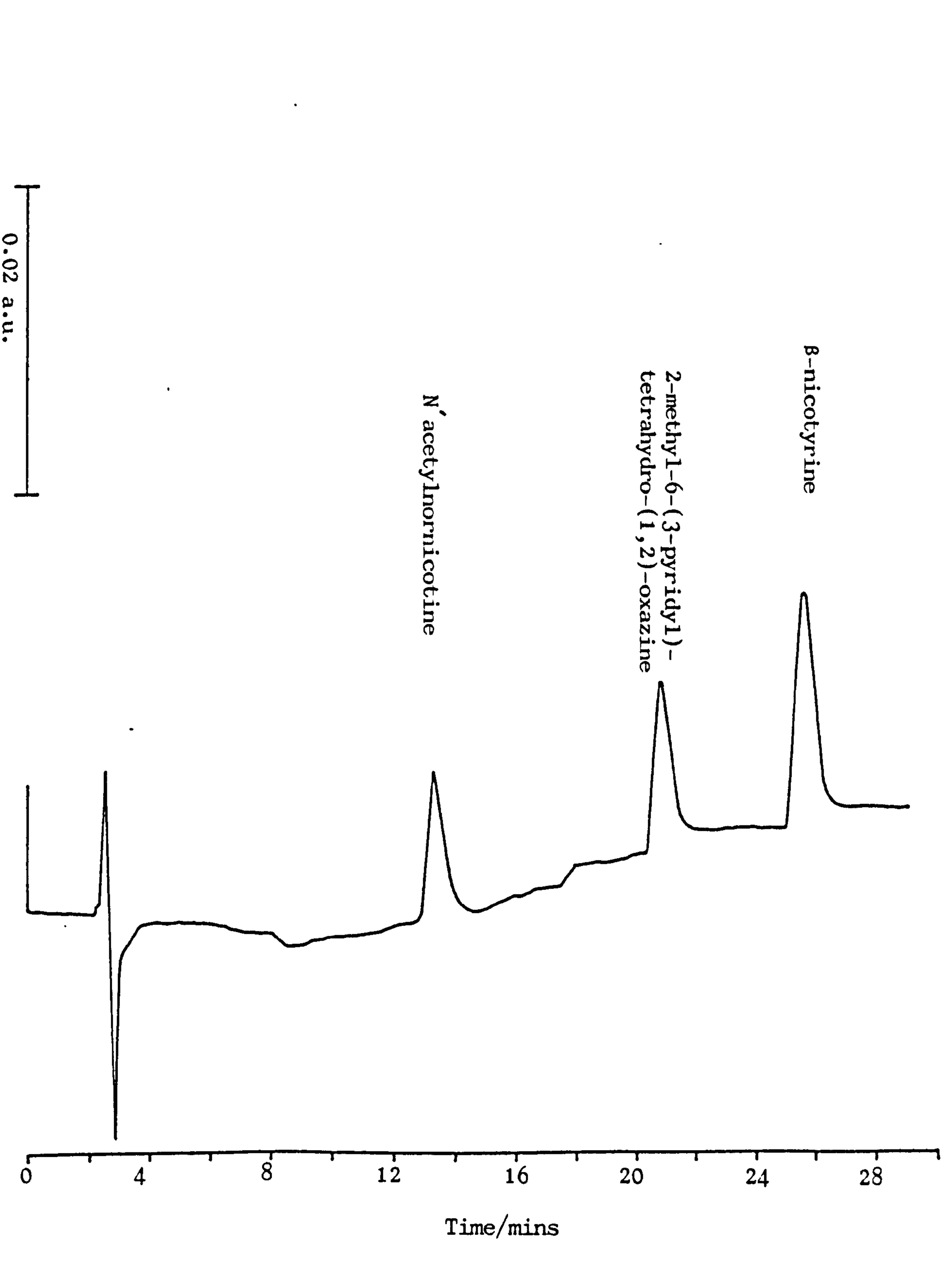
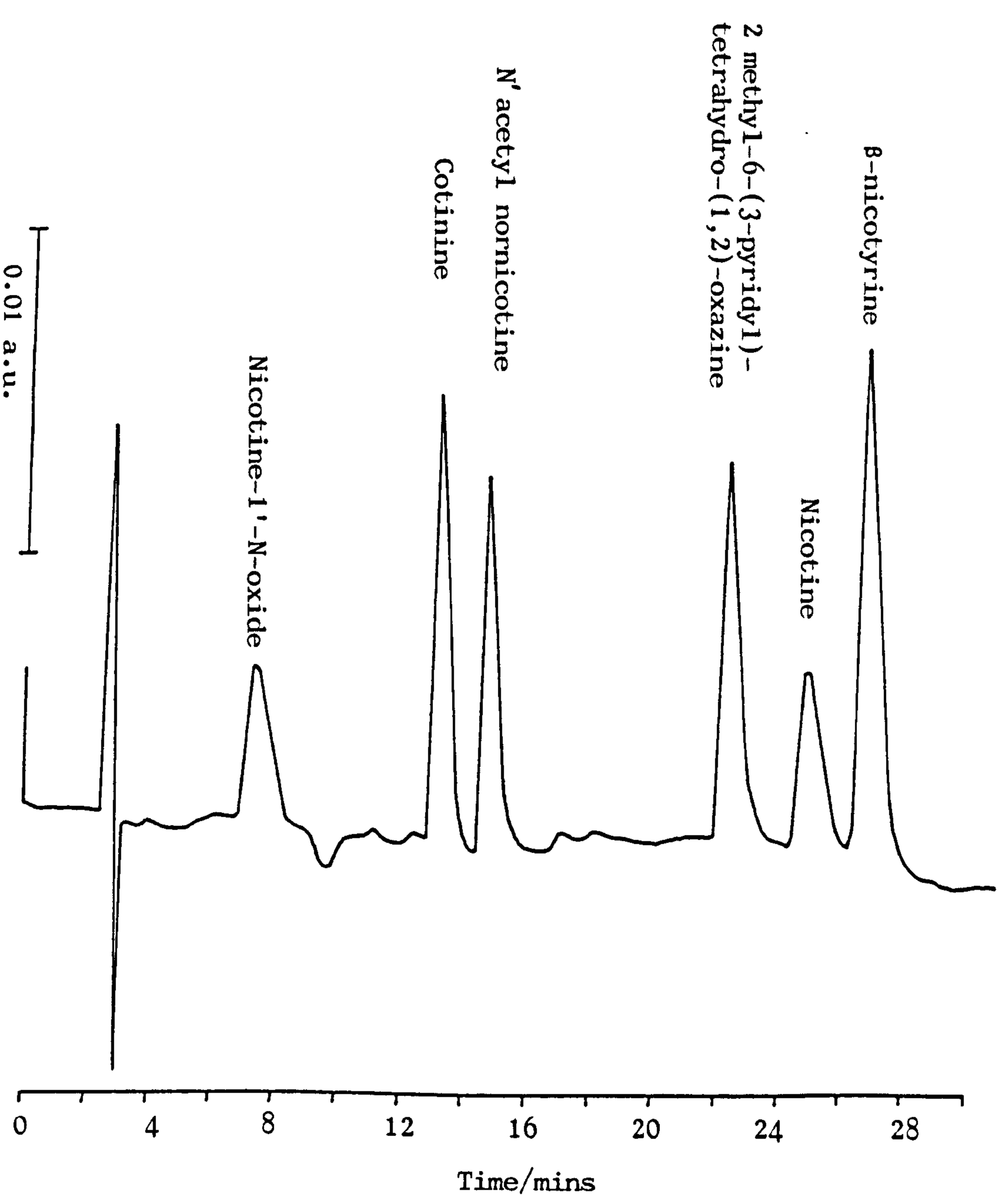


Figure 2.61: Reverse Phase Partition Chromatography of a Standard Mixture containing the components of interest and possible internal standards on a Resolve C_{18} 5 μ Radial PAK cartridge in the RCM-100 using Gradient Elution

Parameters: see figures 2.59 except for:

Eluent: 4 min initial hold at 23% B



required to resolve the two peaks completely. Figure 2.62 and table 2.25 show the variation of the k' values of both 3' hydroxycotinine and nicotine-1'-N-oxide as the percentage of MeOH in the mobile phase is changed. Therefore with 3' hydroxycotinine included in the standard mixture it was necessary to start the gradient programme at 17% MeOH and an initial hold at 17% MeOH for perhaps 3 or 4 minutes was also considered desirable, to gain the best separation possible between nicotine-1'-N-oxide and 3' hydroxycotinine, as shown in figure 2.63.

A chromatogram of nicotine and its three metabolites plus the three possible internal standards (as already stated, β-Nicotyrine was later discarded) is shown in figure 2.64. The programme used was linear and was started at 17% MeOH with an initial hold of 4 mins before rising to 53% MeOH over 19 mins.

2.12 Experimental Equipment

The experimental set-up arrived at through the work described in this chapter and used exclusively during the remainder of this investigation is shown in Plate 1.

The column system finally considered most appropriate was the Resolve C_{18} 5 μ cartridge in the Waters RCM-100 unit and this is shown in detail in Plate 2. (Further details are reported in Chapter 5.)

Plate 1. HPLC equipment arranged for experimental use.

Plate 2. A Waters RCM-100 unit with a Resolve C_{18} 5 μ cartridge.

Table 2.25 and Figure 2.62:
The relationship between the retention time/capacity factor and methanol content of the mobile phase for standard components under reverse phase partition conditions on a Radial PAK Resolve C_{18} 5 μ cartridge

Table 2.25

% Organic Modifier (MeOH)	t_M (mins)	Nicotine-1'-N-oxide		3' Hydroxycotinine	
		t_R(mins)	k'	t_R(mins)	k'
17	2.6	9.14	2.52	11.65	3.48
18	2.6	8.60	2.31	10.57	3.06
20	2.6	7.85	2.02	9.33	2.59
22	2.6	7.00	1.69	8.07	2.10
24	2.6	6.57	1.53	7.36	1.83

Figure 2.62

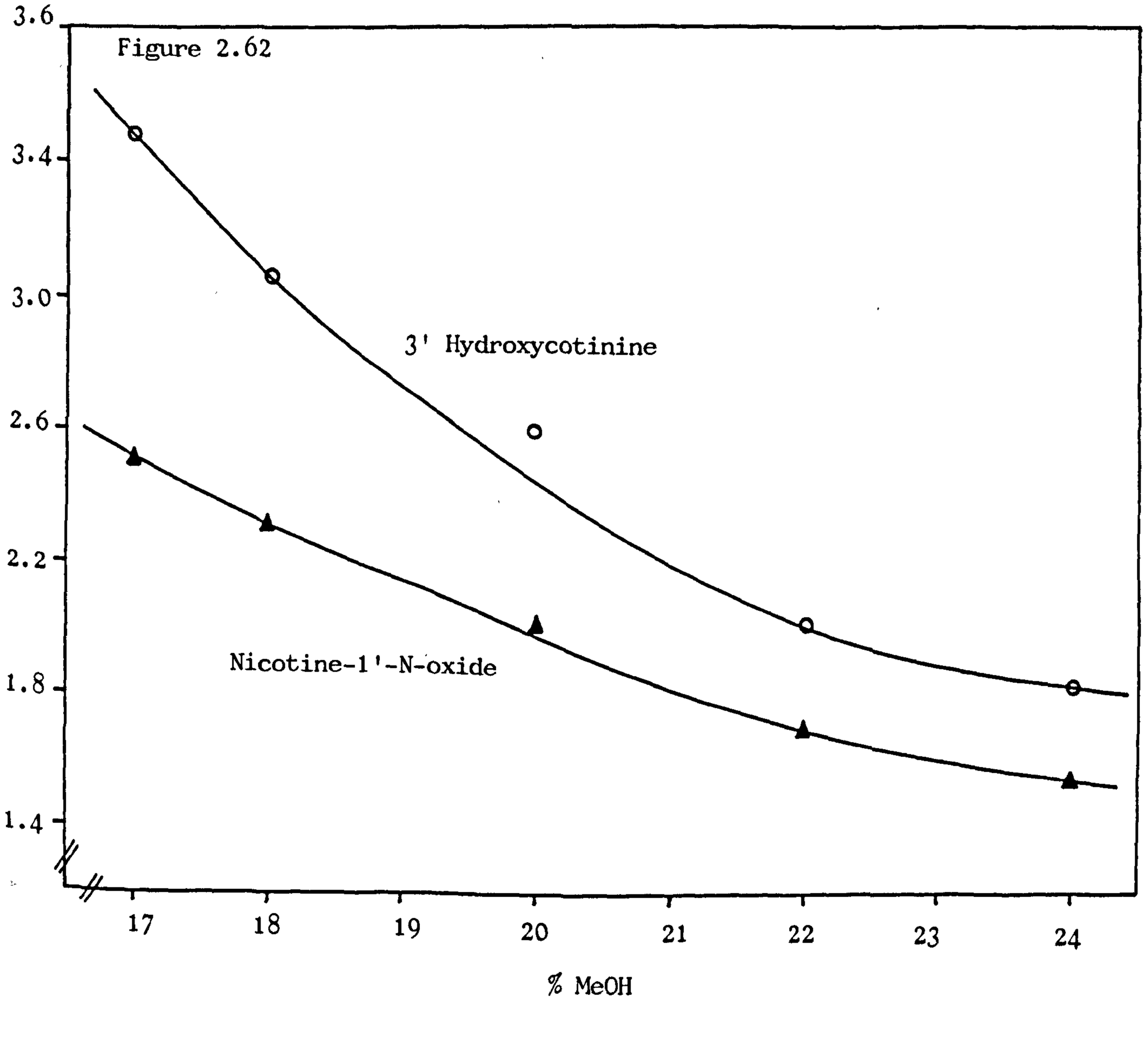
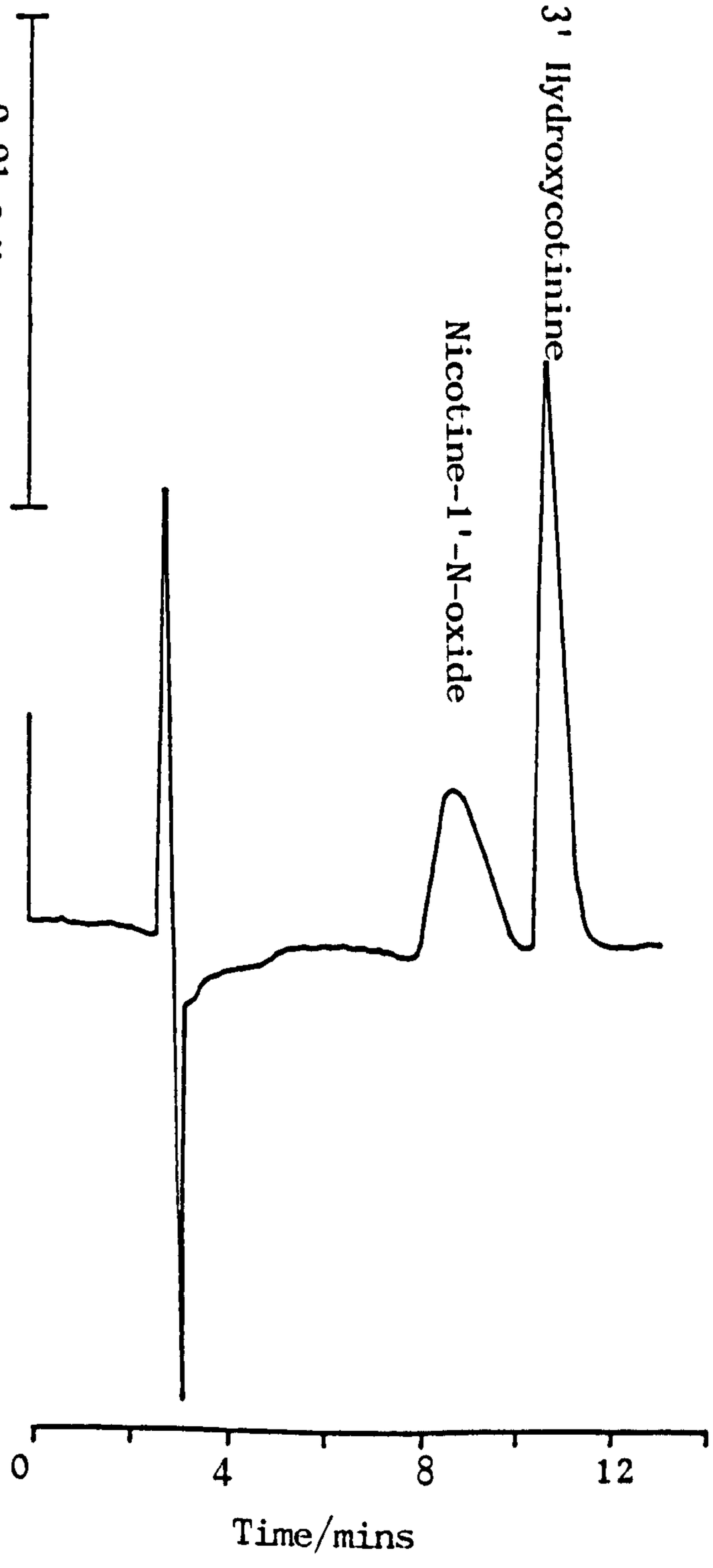


Figure 2.63: Reverse Phase Partition Chromatography of a Standard Mixture of Nicotine-1'-N-oxide and 3' Hydroxycotinine on a Resolve C_{18} 5 μ Radial PAK cartridge in the RCM-100 using Gradient Elution

Parameters: see figures 2.59 except for:

Eluent: 4 min initial hold at 17% B. Gradient programme #6, 17% → 58% B over 15 mins



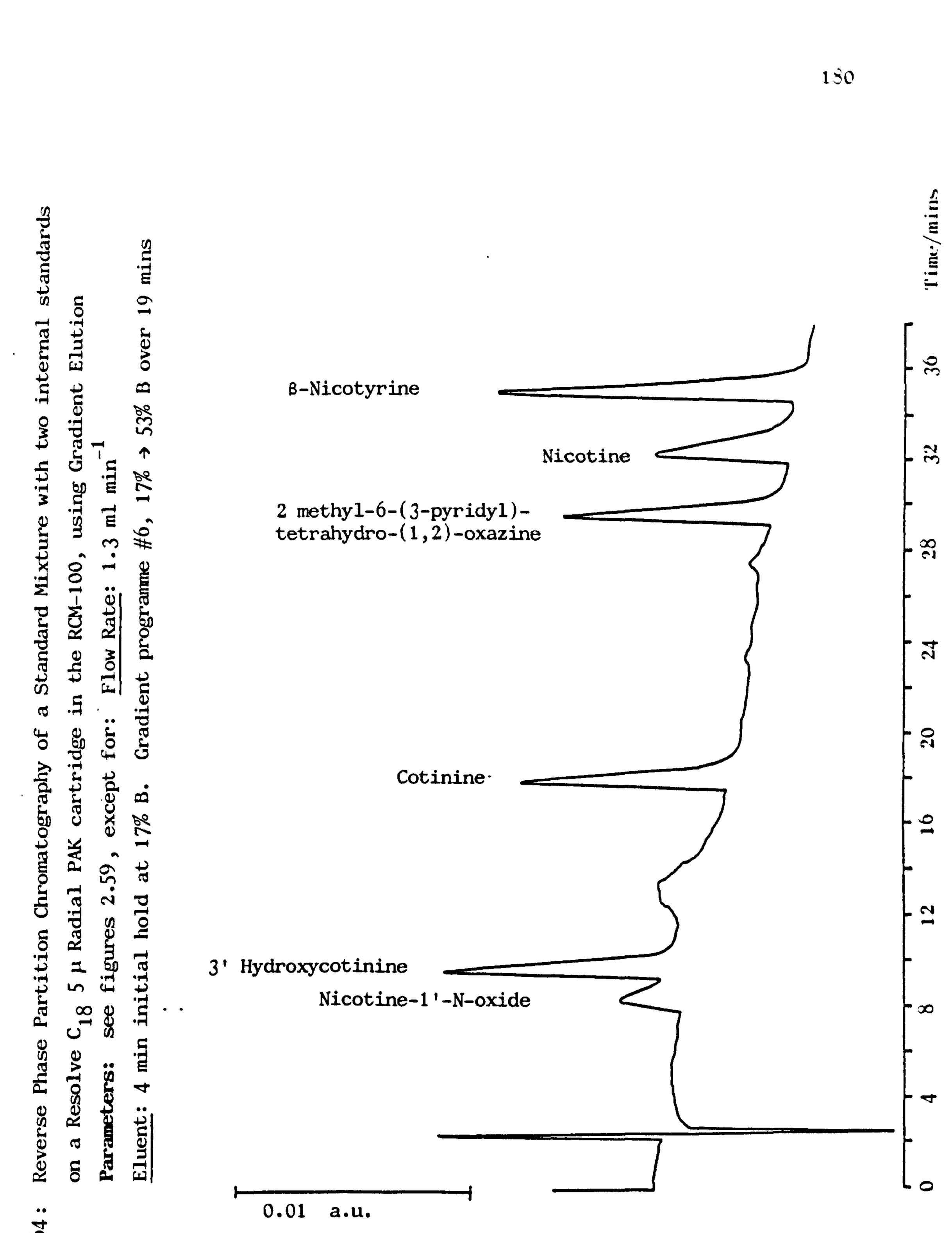
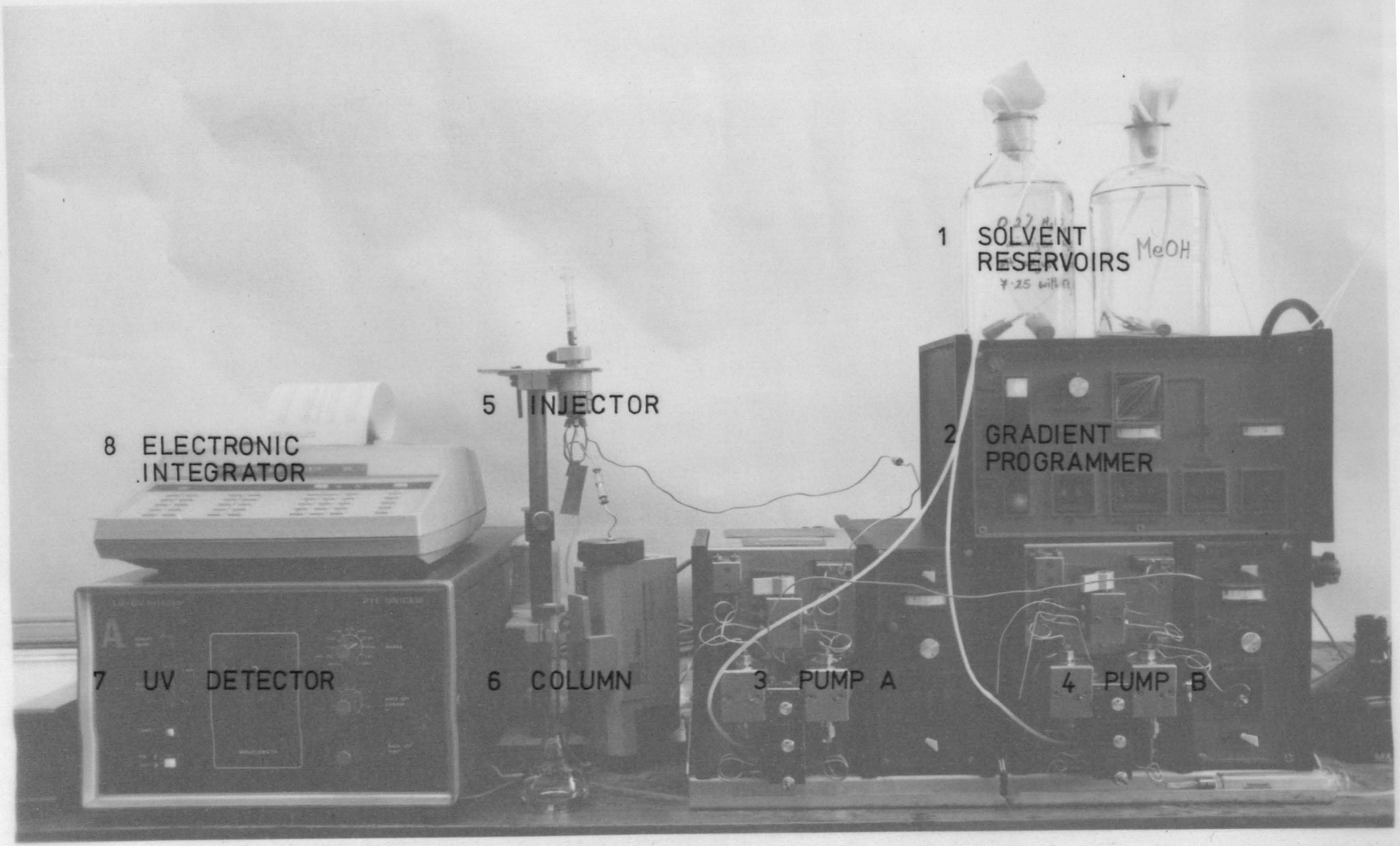
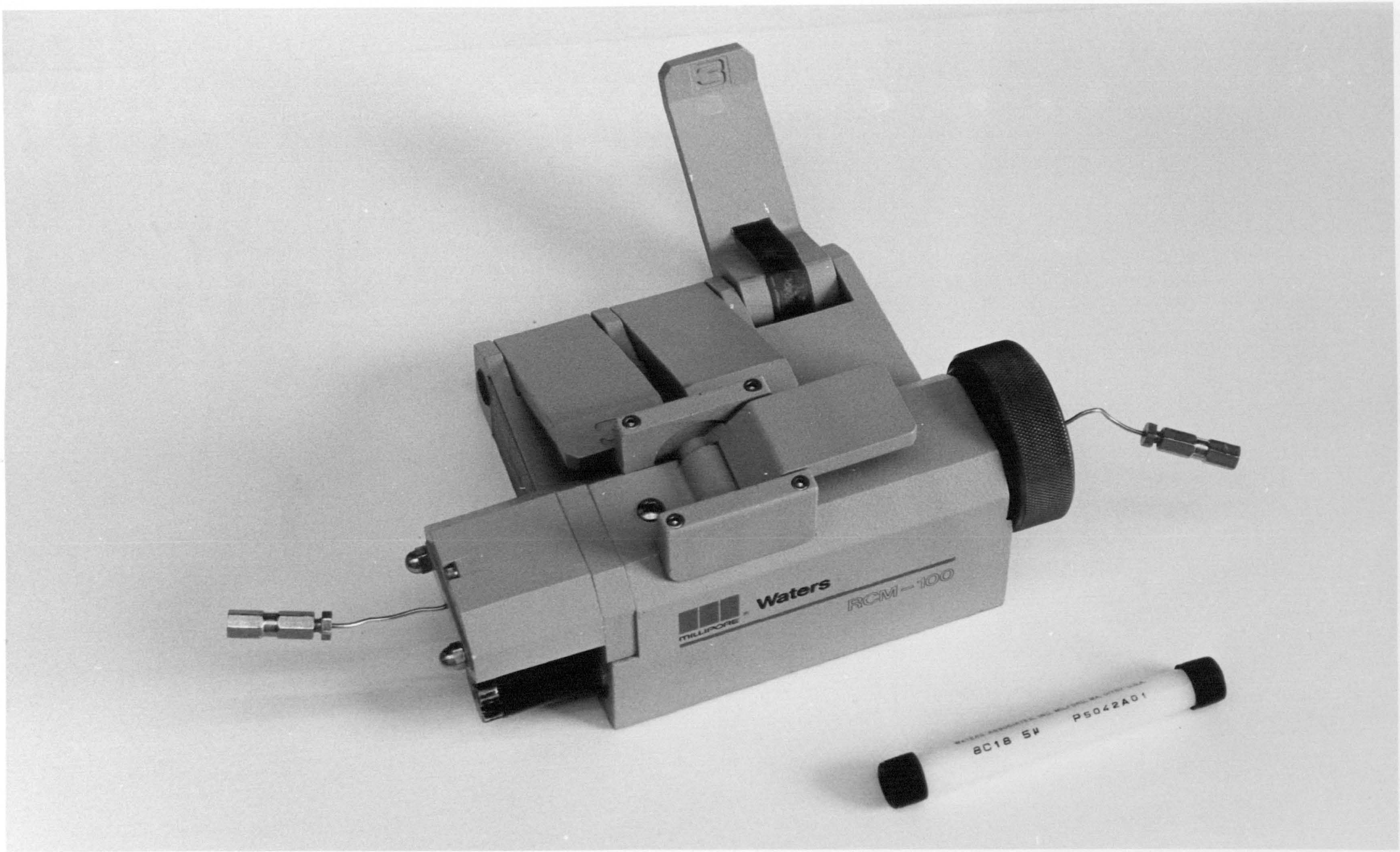


Figure 2.64: Reverse Phase Partition Chromatography of a Standard Mixture with two internal standards on a Resolve C_{18} 5 μ Radial PAK cartridge in the RCM-100, using Gradient Elution

Parameters: see figures 2.59, except for: Flow Rate: 1.3 ml min^{-1}

Eluent: 4 min initial hold at 17% B. Gradient programme #6, 17% → 53% B over 19 mins


1 SOLVENT RESERVOIRS
MeOH
2 GRADIENT PROGRAMMER
3 PUMP A
4 PUMP B
5 INJECTOR
6 COLUMN
7 UV DETECTOR
8 ELECTRONIC INTEGRATOR


Waters
RCM-100
8C18 5µ P5042A01

CHAPTER 3

STATISTICAL ANALYSIS OF THE INSTRUMENT RESPONSE IN THE ANALYSIS OF NICOTINE AND ITS METABOLITES IN STANDARD SOLUTIONS

3.1 Quantitative Analysis [198]

The ultimate objective of this development work is to provide a quantitative method for the analyses of nicotine and its metabolites. As with all quantitative studies, the errors associated with the results are very important. Indeed no quantitative results are of any value unless they are accompanied by some estimate of the errors inherent in them.

In this chapter the repeatability or within-run precision and reproducibility or between-run precision of the method have been examined. Calibration curves have been constructed for each of the standards and the limit of detection calculated. Having gained some information about the experimental errors associated with this analytical method it is hoped to give meaning to the quantitative data obtained, when the method is applied to samples. The calculation of experimental errors also provides a means of comparing the method described here with different methods of analysis or with 'improvements' in this method.

3.2 Repeatability - Within-run Precision of Peak Measurement[198]

To measure the within-run precision, the repeatability, replicate analyses were carried out in succession under exactly the same set of conditions. A standard containing nicotine, cotinine, 3' hydroxy-cotinine and nicotine-1'-N-oxide, at a concentration of 9 $\mu g\ ml^{-1}$, together with the internal standard, 10 $\mu g\ ml^{-1}$, was chromatographed ten times consecutively. A typical trace is shown in figure 3.1.

To carry out the replicate runs, the Waters Gradient System was used with a Radial Pak C_{18} cartridge. Chromatography was carried out using the following conditions: Programme # 6, 5 min initial hold at 18% MeOH, 18% → 58% B over 19 mins, at a flow rate of 1.2 $ml\ min^{-1}$. The UV detector signal was fed to both a chart recorder and an electronic integrator which were connected in parallel. Integration parameters were set after preliminary investigations and were then programmed into the integrator and remained unchanged during the course of the experiment.

From each standard run, peak areas were obtained from the integrator and peak heights were measured manually from the chromatograms. Peak area ratios and peak height ratios (standard/IS) were calculated. The data for the replicate injections is presented in table 3.1.

The repeatability was determined using each of the different measurement parameters for each of the standards. The results have been reported as a mean value calculated for the ten replicate analyses and the degree of spread about this mean value, the standard deviation, s. The relative standard deviation (RSD) given by $100\ s/\bar{x}$ is the relative error, expressed as a percentage. The RSD was also reported

Parameters:

Resolve C_{18} 5 μ cartridge (10 cm x 8 mm ID) in a Waters Radial Compression Module

Liquid A: 0.2% H_3PO_4 adjusted to pH 7.25 with Et_3N.

Liquid B: MeOH

Detection: UV at 260 nm into 10 mV f.s.d.

Sensitivity: 0.04 a.u.f.s.

Sample Size: 100 μl

Flow Rate: 1.2 ml min^{-1}

Eluent: 5 min initial hold at 18% B. Prog. #6, 18%-58% B over 19 mins.

Sample: Standard containing 9 μg ml^{-1} (≡0.9 μg injected) Nicotine-1'-N-oxide, 3' Hydroxy-cotinine, Cotinine and Nicotine and 10 μg ml^{-1} IS (≡ 1 μg injected) in H_2O

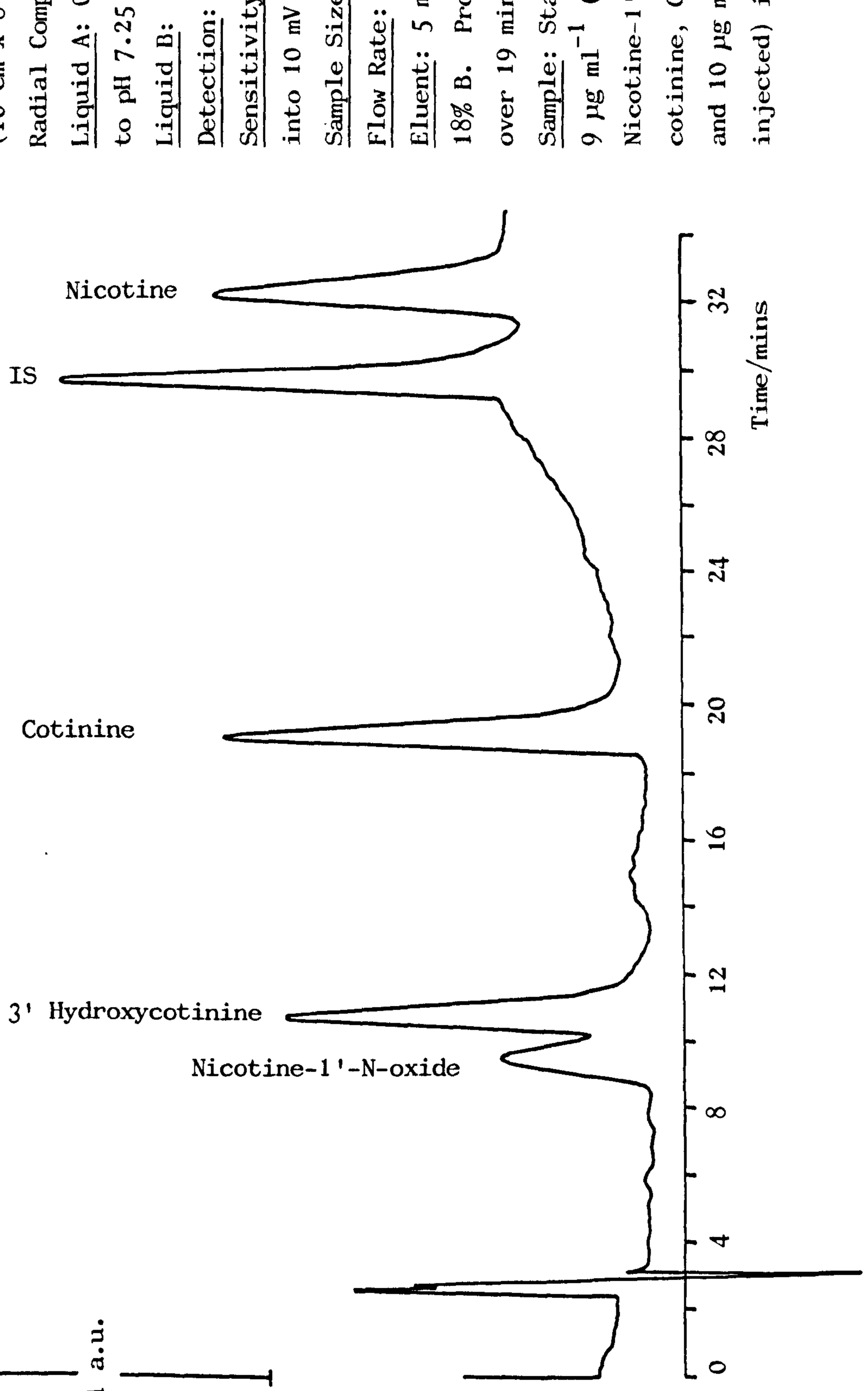


Figure 3.1: A Typical Chromatogram obtained during the Within-run Precision Determination

TABLE 3.1: Peak Measurement Data for Replicate Injections of a 9.μg ml^{-1} standard mixture (n = 10)

Measurement Parameter	Run Number	Nicotine-1'-N-oxide	3' Hydroxy-cotinine	Cotinine	Internal Standard	Nicotine
Peak Height (mm)	1	21.5	57.5	61.0	65.0	44.5
	2	22.0	60.0	62.5	67.0	45.5
	3	21.0	56.5	65.0	70.0	45.0
	4	20.0	58.0	64.0	69.5	46.0
	5	20.0	55.5	64.0	69.5	44.5
	6	22.5	54.5	63.0	66.5	45.0
	7	21.0	54.0	61.5	66.5	45.5
	8	20.5	57.0	61.0	67.5	45.5
	9	20.0	60.5	64.0	67.5	45.5
	10	19.5	60.5	63.5	66.0	44.5
Peak Height Ratio	1	0.3307	0.8846	0.9384	-	0.6846
	2	0.3283	0.8955	0.9328	-	0.6791
	3	0.3000	0.8071	0.9285	-	0.6428
	4	0.2877	0.8345	0.9208	-	0.6618
	5	0.2877	0.7985	0.9208	-	0.6402
	6	0.3383	0.8195	0.9473	-	0.6766
	7	0.3157	0.8120	0.9248	-	0.6842
	8	0.3037	0.8444	0.9037	-	0.6740
	9	0.2962	0.8962	0.9481	-	0,6740
	10	0.2954	0.9166	0.9621	-	0.6742
Peak Area (counts)	1	340800	788900	1079000	1135700	1018300
	2	276800	783100	1026200	985000	999100
	3	250800	740500	1021900	929800	969200
	4	347500	797300	1130600	1168000	1020100
	5	362200	797800	1158900	1174900	1020100
	6	365700	817600	1201700	1179100	1026400
	7	343700	792900	1126400	1165600	1019100
	8	340600	785000	1077200	1098700	1013900
	9	382400	827200	1202400	1432300*	1059100
	10	364100	800900	1163400	1176100	1026400
Peak Area Ratio	1	0.3000	0.6946	0.9500	-	0.8966
	2	0.2809	0.7950	1.0417	-	1.0142
	3	0.2697	0.7964	1.0990	-	1.0424
	4	0.2975	0.6826	0.9679	-	0.8733
	5	0.3083	0.6790	0.9863	-	0.8682
	6	0.3101	0.6933	1.0191	-	0.8704
	7	0.2948	0.6802	0.9663	-	0.8743
	8	0.3099	0.7145	0.9804	-	0.9228
	9	0.2669	0.5775	0.8394	-	0.7394
	10	0.3095	0.6809	0.9892	-	0.8727

as it is very convenient in the comparison of precision estimates of results which have different units or magnitudes. All results have been presented in table 3.2(a). From table 3.2(a) it can be seen that peak height measurement gives the lowest mean RSD for all analyte peaks and that both peak height and peak height ratio measurement prove to be superior to those of peak area or peak area ratio. It was expected that peak area would be a better method of measurement than peak height as the standards were chromatographed using a gradient system and peak height measurements would be more prone to slight variations in the gradient from one run to another and also that peak area ratio would be superior to peak area measurements.

On close examination of table 3.1 which presents the raw data obtained from this experiment, an exceptionally high value of peak area was recorded for the internal standard peak (Run 9). If this value is an 'outlier' then it may contribute to the relatively high values of the standard deviation, s, and RSD for both peak area and peak area ratio measurements. Since the discussion of the precision of the method depends on these final values, it is important to assess the suspect measurement and whether it can be rejected. To assess the suspect measurement, a statistical test known as Dixon's Q Test was applied. It compares the difference between the suspect value and the measurement nearest to it in size, with the difference between the highest and lowest measurements. The ratio of these differences (without regard to sign) is the Q value.

$$Q = \frac{|\text{suspect value} - \text{nearest value}|}{\text{largest value} - \text{smallest value}}$$

If the calculated value of Q exceeds the critical value, the suspect value is rejected.

TABLE 3.2(a): Statistical Data for Repeat Injections of a 9 μg ml^{-1} standard mixture (n = 10)

Measurement Parameter		Nicotine-1'-N-oxide	3' Hydroxy-cotinine	Cotinine	Internal Standard	Nicotine	Mean RSD for all Analyte Peaks
Peak Height	Mean (mm)	20.80	57.4	62.95	67.50	45.15	
	± SD (mm)	0.98	2.4	1.4	1.7	0.5	
	RSD (%)	4.7	4.1	2.2	2.5	1.2	2.9
Peak Height Ratio	Mean	0.31	0.85	0.93	-	0.67	
	± SD	0.02	0.04	0.02	-	0.02	
	RSD (%)	6.0	5.1	1.8	-	2.4	3.8
Peak Area	Mean (counts)	337400	793100	1118800	1144500	1017200	
	± SD (counts)	41500	23100	65800	133600	22600	
	RSD (%)	12.3	2.9	5.9	11.7	2.2	7.0
Peak Area Ratio	Mean	0.30	0.70	0.98	-	0.90	
	± SD	0.02	0.06	0.07	-	0.08	
	RSD (%)	5.6	8.9	6.8	-	9.4	7.7

$$Q = |1432300 - 1179100|/1432300 - 929780$$

$$= 253200 / 502520$$

$$= 0.5039$$

The critical value of Q, for a sample size of 10, is 0.464 (P = 0.05), therefore, the suspect value can be rejected.

Table 3.2(b) shows the re-calculated values of mean, standard deviation and RSD for the peak area and peak area ratio measurement parameters where the suspect value has been rejected.

The peak height measurement method still yields the highest within-run precision (mean RSD = 2.9%), and both the peak height and peak height ratio measurement methods result in higher within-run precision than peak area or peak area ratio measurement methods.

The within - run precision of the two external methods of calibration, peak area (RSD = 6.3%) and peak height (RSD = 2.9%) were expected to be equivalent. Janik[199] found that manually measured peak heights and electronically integrated peak areas obtained from a GC output were equivalent with respect to precision, even though peak height is sensitive to instrumental and operational variations whereas peak area suffers less from this disadvantage. McCoy et al.[200] have also reported that peak area measurements result in precision values equivalent or superior to those obtained for peak height measurements only if the peaks were 'well-behaved' or were not subject to chromatographic interference. Where peaks were poorly resolved from the solvent front or an earlier eluting peak, or if the baseline was not well established, peak height measurements appeared to provide more precise quantitation. It must be noted that McCoy et al.[200] carried out the experiments with isocratic elution and no similar

TABLE 3.2(b): Statistical Data, with Peak Area and Peak Area Ratio as the measurement parameters, for repeat injections of a 9 μg ml^{-1} standard mixture, recalculated after rejection of an outlier

Measurement Parameter		Nicotine-1'-N-oxide	3' Hydroxy-cotinine	Cotinine	Internal Standard	Nicotine	Mean RSD for all Analyte Peaks
Peak Area	Mean (counts)	337400	793100	1118800	1112600	1017200	
	± SD (counts)	41500	23100	65800	92600	22600	
	RSD (%)	12.3	2.9	5.9	8.3	2.2	6.3
Peak Area Ratio	Mean	0.30	0.71	1.00	-	0.92	
	± SD	0.01	0.05	0.05	-	0.07	
	RSD (%)	4.8	6.8	4.6	-	7.3	5.9

experiments under gradient elution conditions were attempted. However, their conclusions are in general agreement with the results of this experiment and the superiority of the peak height measurements with the exception of those for 3' hydroxycotinine.

A significant contribution to the much lower precision values reported when peak area measurements were used may be attributed to erratic integration of the peaks. The integrator parameters were optimized before the start of the experiment, however, the use of the gradient run, with a changing baseline, made this a difficult problem to resolve.

An internal standard was included with the intention of improving the precision of the experiment. The results show that this was found to be true only when peak area was the measurement parameter.

Haefelfinger [201] derived the following formula as an aid to be used when inclusion of the internal standard is under consideration.

$$RSD_{IS} < 2\,r.\,RSD_{analyte}$$

where RSD_{IS} = the relative standard deviation of the area (or height) of the IS

$RSD_{analyte}$ = the relative standard deviation of the area (or height) of the analyte

and r = the correlation coefficient which is in the range $0 < r \leq 1$ since a positive correlation must exist between the area (or height) of the IS and that of the analyte.

If the above relationship is true, the inclusion of an IS will result in an improvement in the precision of the method.

Applying this relationship, using the RSD values for the analyte and internal standard peaks, quantitated both by height and area,

predicted correctly whether inclusion of an IS would result in an improvement in the precision of the method, in every case where the imposed conditions were satisfied. The overall result, with peak height as the measurement parameter, given by the mean RSD for all analyte peaks was a decrease in the precision of the method from 2.9% to 3.8% when the IS was included, whereas with peak area as the measurement parameter there was an improvement in the overall precision of the method from 6.3% to 5.9%.

To summarize, the best precision was a result of quantitation by peak height measurement and both peak height measurement parameters give better within-run precision than the corresponding peak area measurement parameters. The unexpectedly poor precision recorded for both peak area measurement parameters may be caused by the uncertainty involved in the recognition of the start and end points of the peaks.

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3.3 Reproducibility - Between-run Precision of Peak Measurement

When the analysis is carried out on different occasions, the conditions may be different, and hence a greater spread of the results may be found in this case. The data obtained from such an experiment would reflect the between-run precision of the method, also known as the reproducibility.

In order to assess the between-run precision a series of standard solutions (with concentrations 1, 2, 4, 5, 7, 9, 10 and 20 $\mu g\ ml^{-1}$ each of nicotine-1'-N-oxide, 3' hydroxycotinine, cotinine and nicotine

plus the internal standard, 10 μg ml^{-1}) were chromatographed over a one month period - the series being repeated 12 times in all. A typical series of chromatograms are shown in figure 3.2, (a)-(h). As in the previous experiment, the detector signal was fed to both a chart recorder and an electronic integrator which were connected in parallel. Peak heights were measured manually and peak area recorded from the integrator for each run and peak height ratios and peak area ratios were calculated. All the data are presented in tables 3.3-3.6. In order to determine the between-run precision and also compare the overall precision of each method of quantitation, the mean, SD and RSD were determined separately for each standard component at each concentration level for the four different methods of quantitation. The calculated RSDs were averaged over the entire concentration range for each analyte and are reported in table 3.7. Also reported in table 3.7 are the RSD values averaged over all concentrations and analytes for each quantitation method.

In terms of reproducibility, the peak height ratio measurement parameter was found to give the best overall result (4.9%) and the peak area measurement parameter the poorest (7.0%). The between-run precision was expected to be inferior to the within-run precision and the experimental results for all methods of quantitation show this to be true. Having noted the problem, in the measurement of peak area, concerning the uncertainty in recognition of the start and end-points of the peaks, special attention was paid to the integration parameters which were again optimized before the initial standard runs and remained constant during the course of the experiment. The two external calibration methods, peak area and peak height,

Figure 3.2: Typical Chromatograms obtained by injection of a series of calibration standards

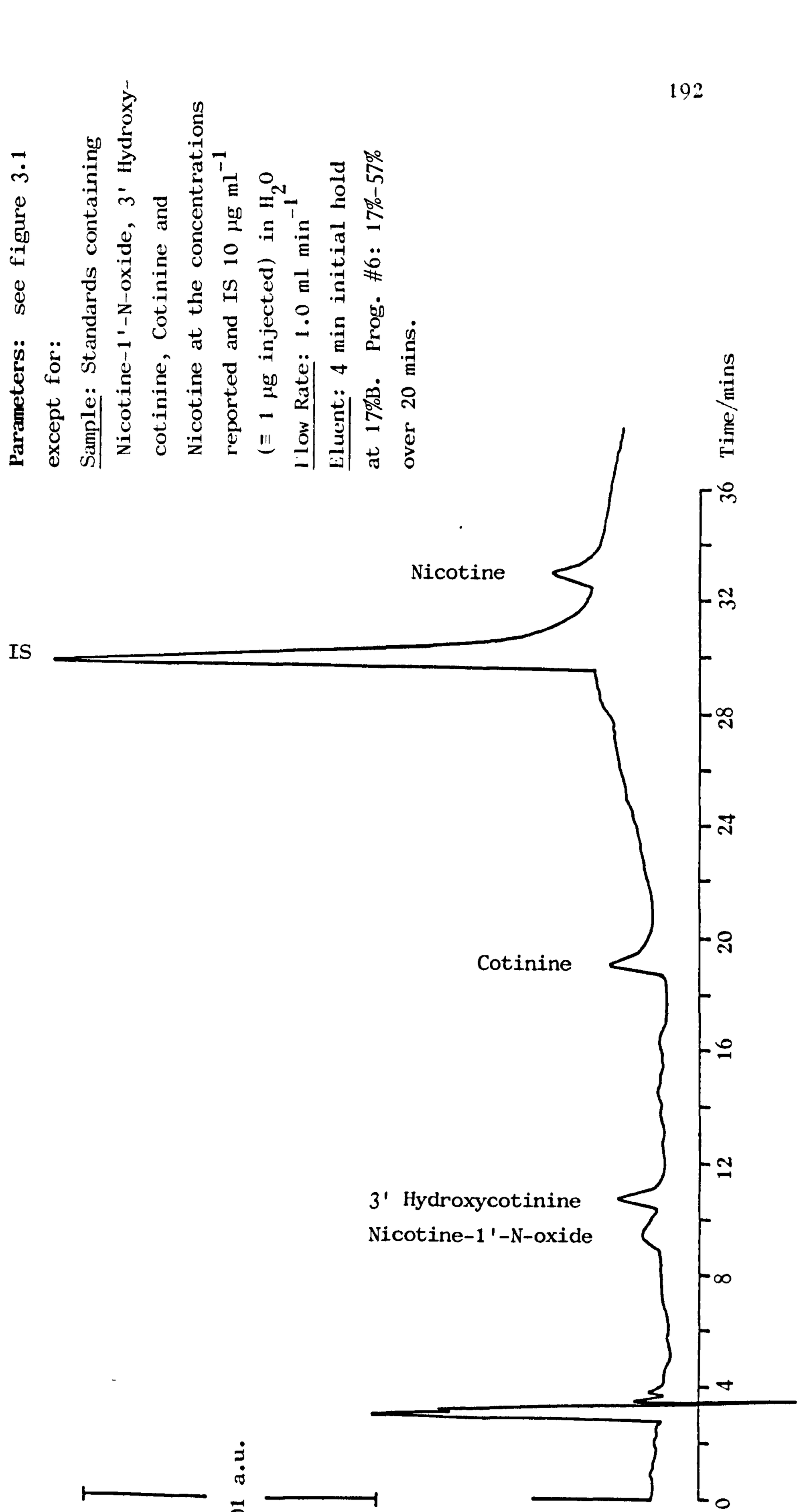
(a) 1 $\mu g\ ml^{-1}$ standard

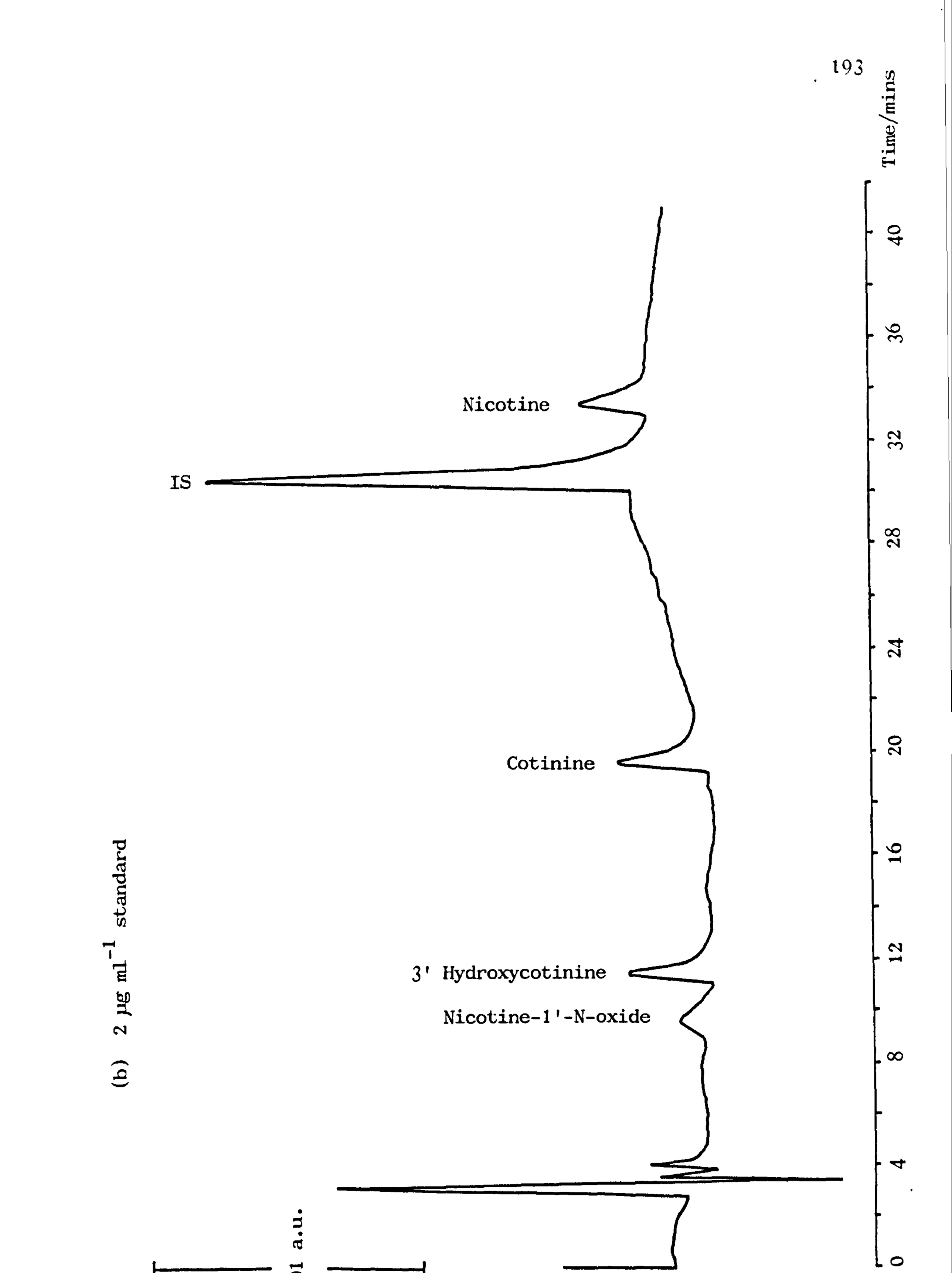
Parameters: see figure 3.1 except for:

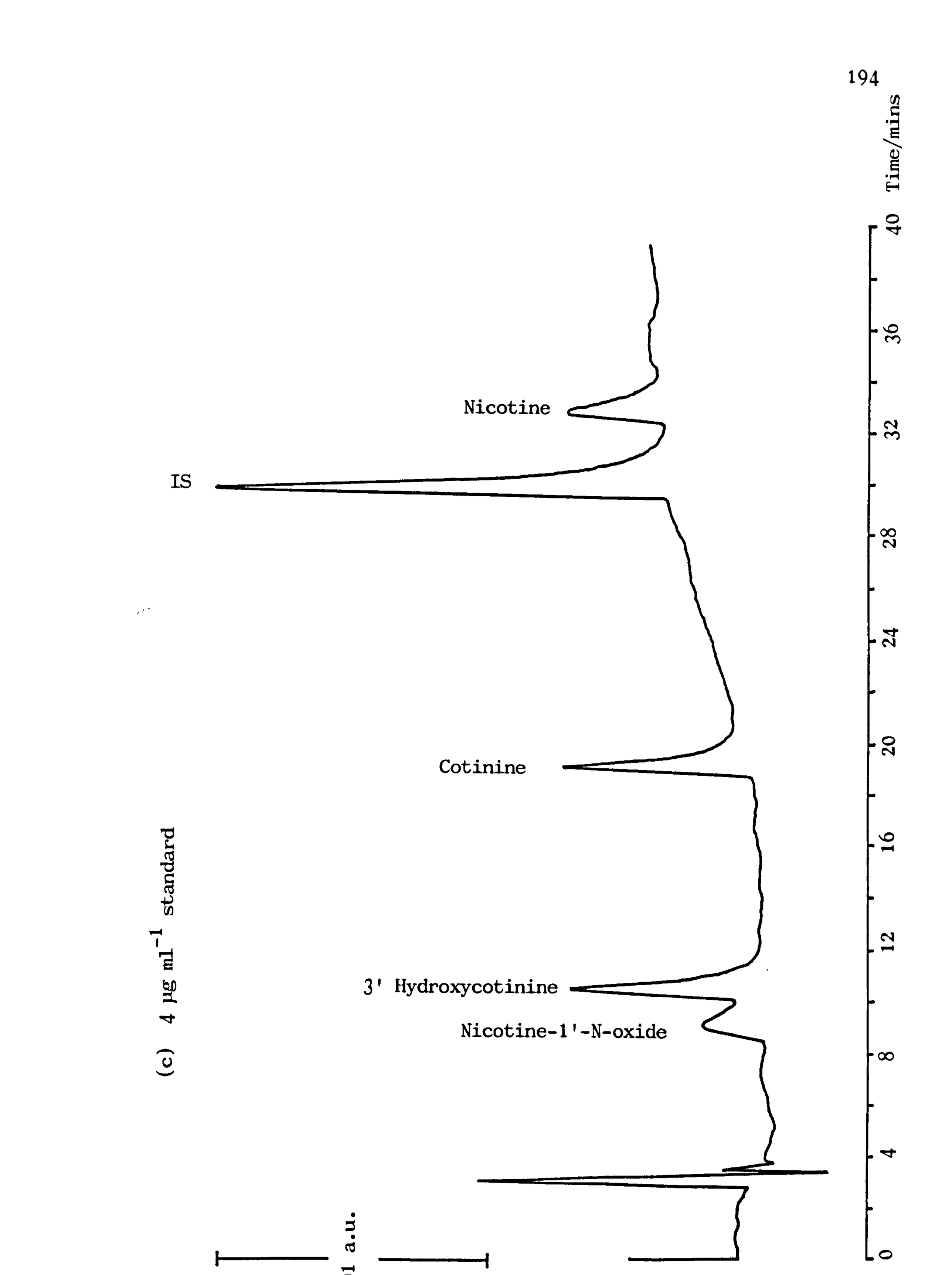
Sample: Standards containing Nicotine, Cotinine and cotinine-1'-N-oxide, 3' Hydroxy-Nicotine at the concentrations reported and IS 10 $\mu g\ ml^{-1}$ ($\equiv$ 1 μg injected) in H_2O

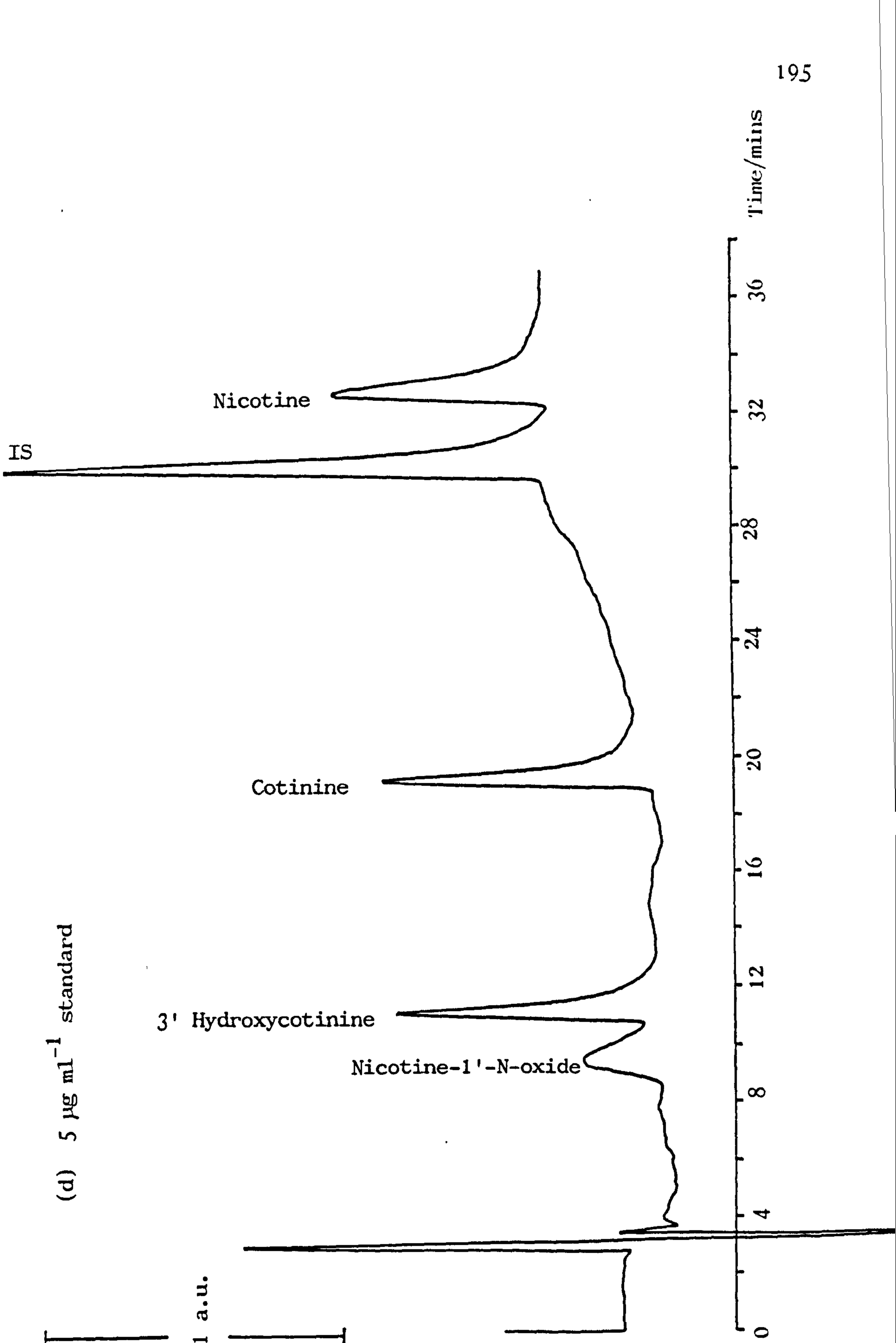
Flow Rate: 1.0 ml min^{-1}

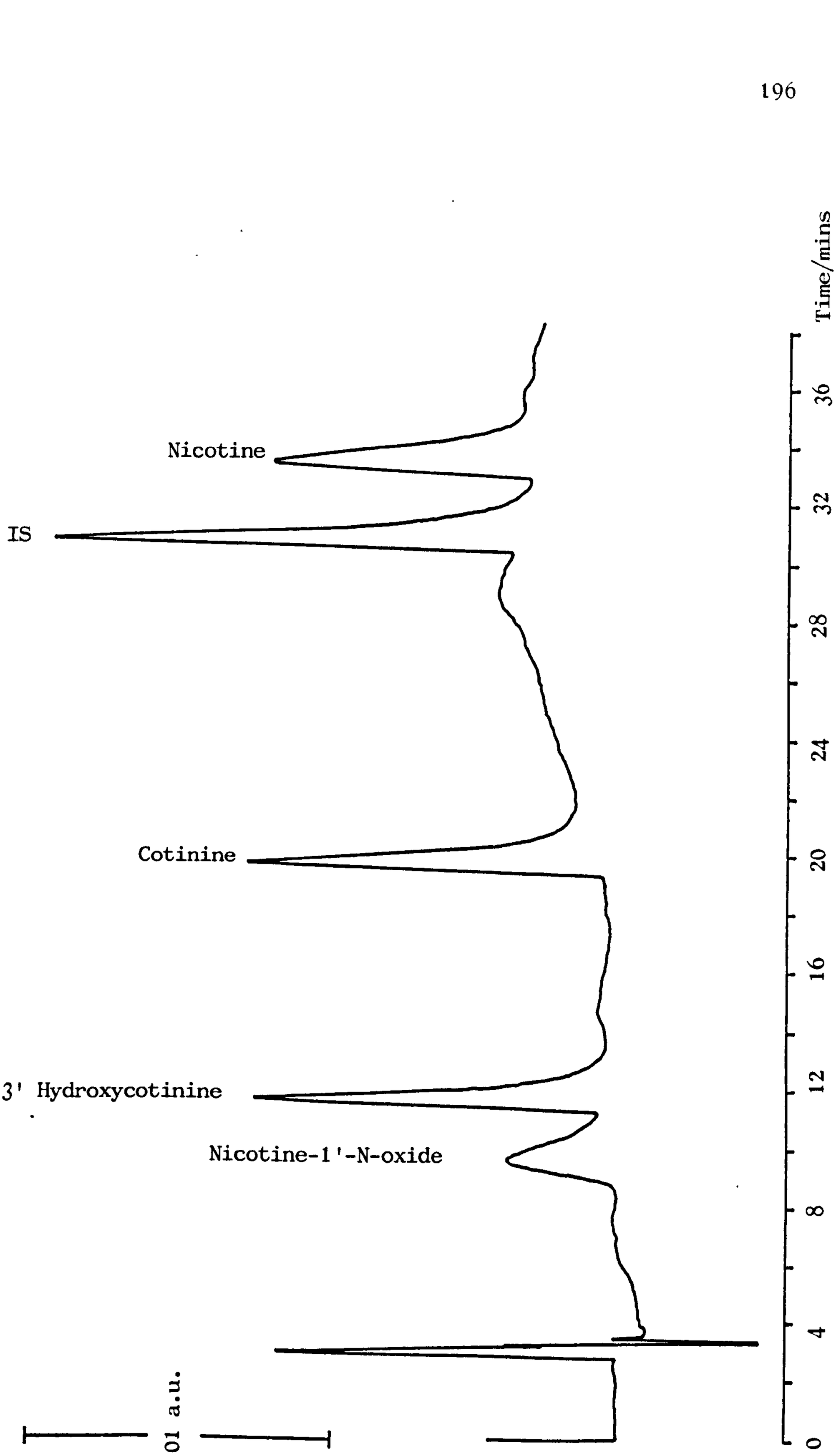
Eluent: 4 min initial hold at 17%B. Prog. #6: 17%-57% over 20 mins.

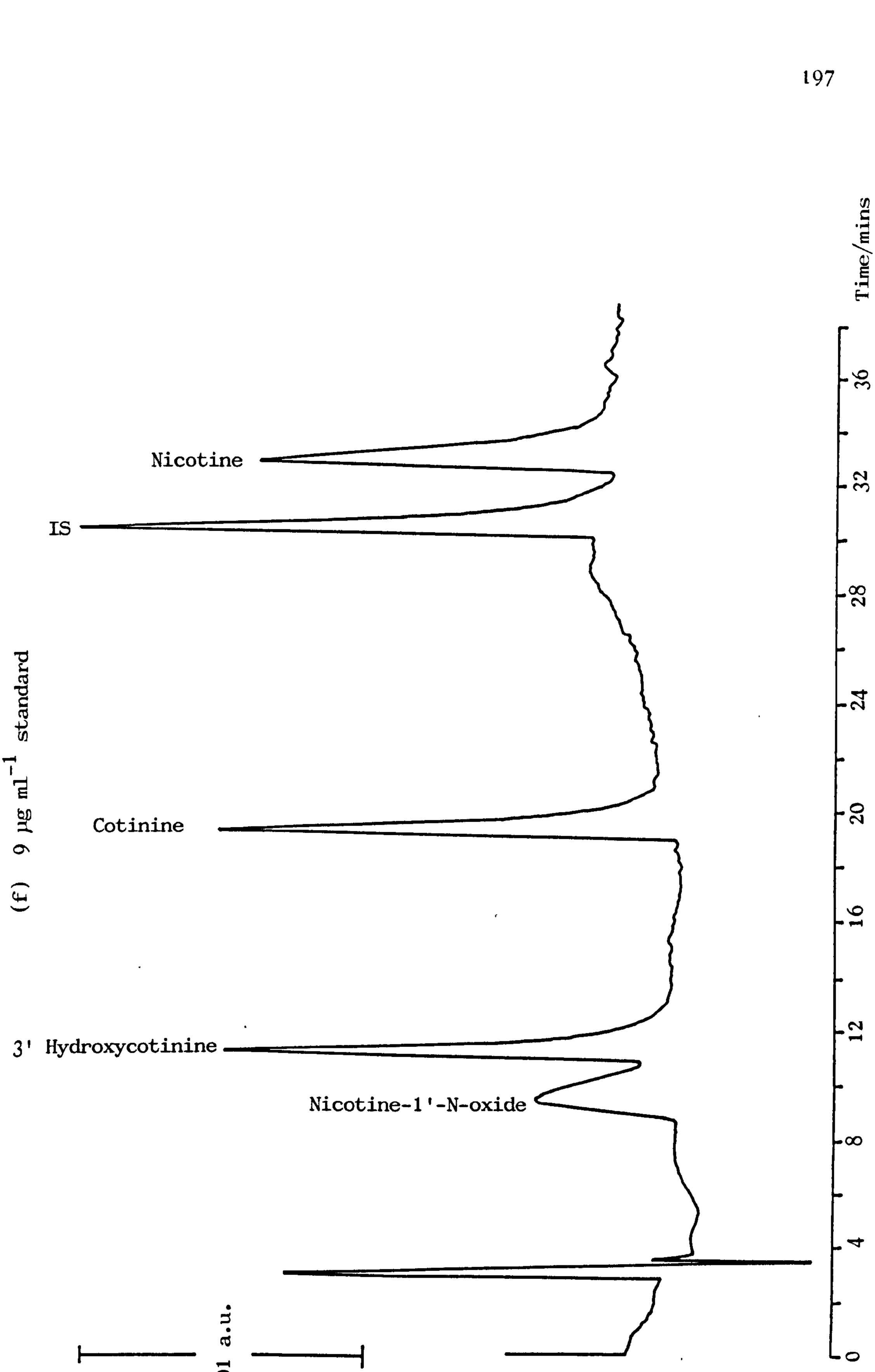


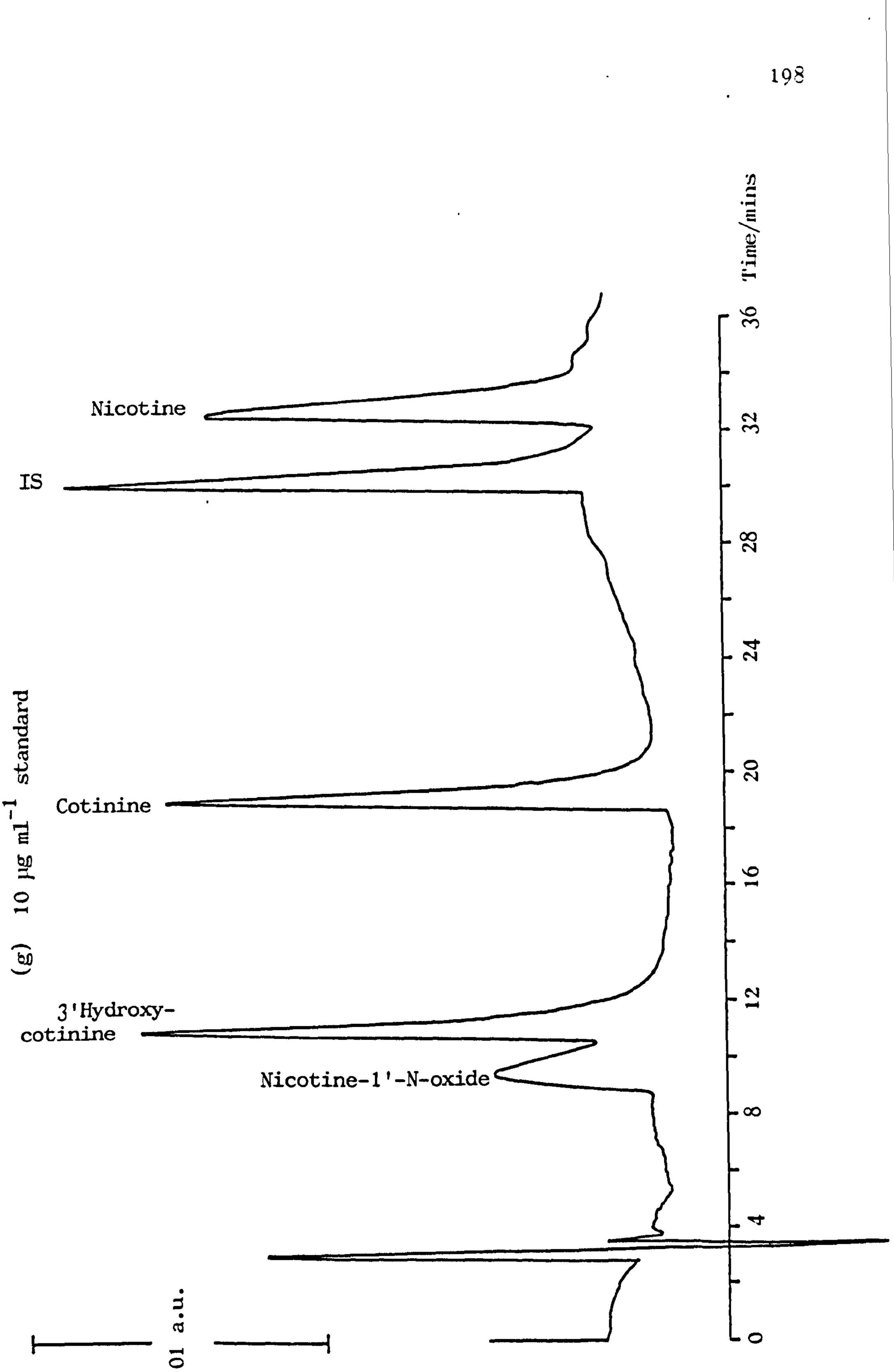

Time/mins
40
36
32
28
24
20
16
12
8
4
0
Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
(b) 2 µg ml^{-1} standard
0.01 a.u.


Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
(c) 4 μg ml^{-1} standard
0.01 a.u.
Time/mins
40
36
32
28
24
20
16
12
8
4
0


Time/mins
36
32
28
24
20
16
12
8
4
0
Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
(d) 5 μg ml^{-1} standard
0.01 a.u.


Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
(e) 7 µg ml-1 standard
0.01 a.u.
Time/mins
36
32
28
24
20
16
12
8
4
0


Time/mins
36
32
28
24
20
16
12
8
4
0
(f) 9 µg ml-1 standard
Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
0.01 a.u.


Time/mins
36
32
28
24
20
16
12
8
4
0
Nicotine
IS
Cotinine
3'Hydroxy-
cotinine
Nicotine-1'-N-oxide
(g) 10 µg ml^{-1} standard
0.01 a.u.

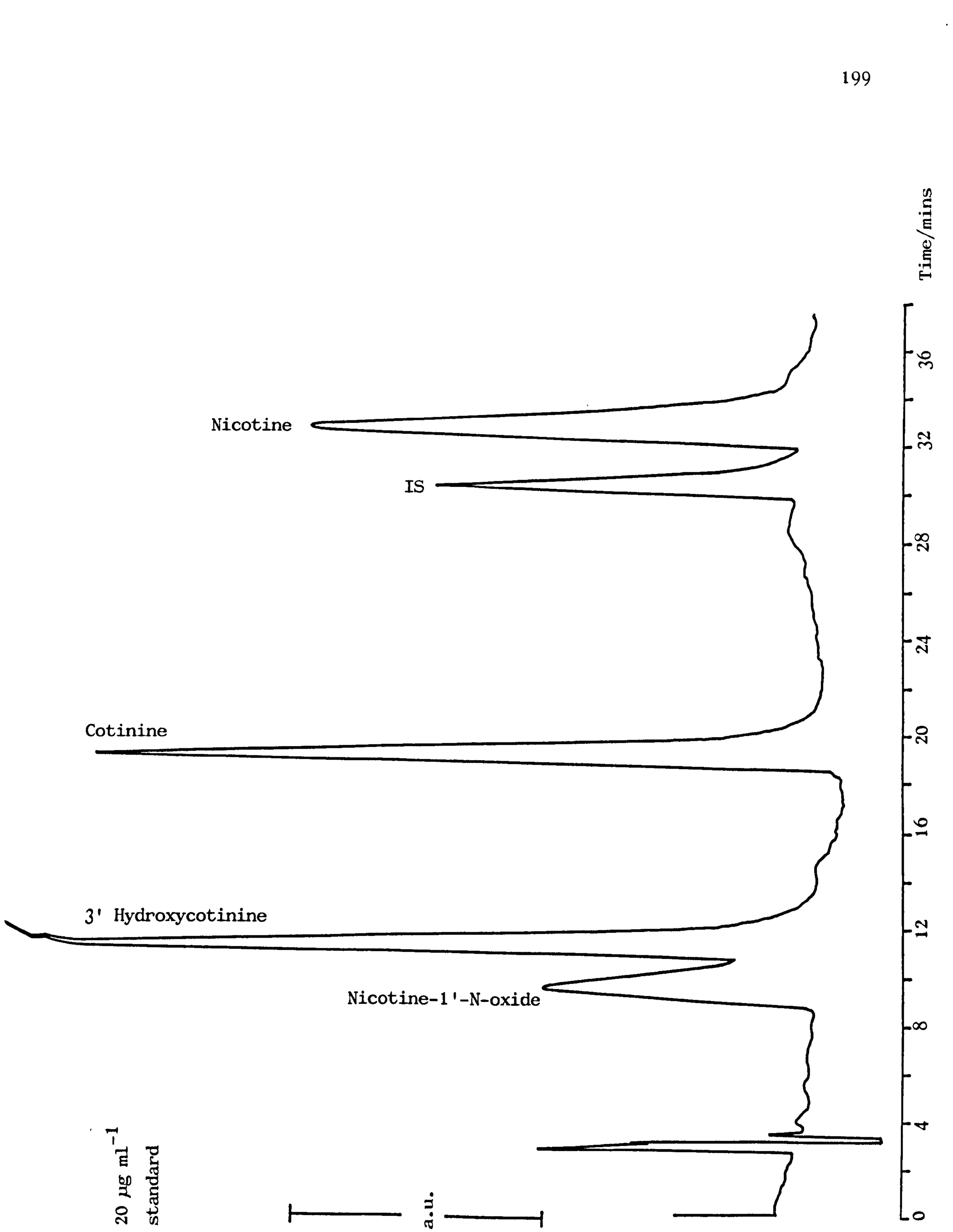

Time/mins
36
32
28
24
20
16
12
8
4
0
Nicotine
IS
Cotinine
3' Hydroxycotinine
Nicotine-1'-N-oxide
0.01 a.u.
(h) 20 μg ml^{-1} standard

TABLE 3.3: Experimental Data for Nicotine-1'-N-oxide over the concentration range 1-20 μg ml^{-1} (n = 12)

X	Y				IS	
Concn. μg ml^{-1}	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	51.5	0.6866	1005900	0.7814	75.0	1287400
10.0	28.0	0.3684	498400	0.3679	76.0	1354600
9.0	24.0	0.2944	576400	0.4393	81.5	1312200
7.0	21.5	0.2514	520500	0.3958	85.5	1315100
5.0	12.0	0.1548	360800	0.2958	77.5	1220200
4.0	11.5	0.1256	177100	0.1428	91.5	1240600
2.0	5.5	0.0705	79900	0.0655	78.0	1221100
1.0	3.5	0.0406	33500	0.0242	86.0	1382900
20.0	55.0	0.7236	973500	0.8615	76.0	1130100
10.0	26.0	0.3058	532500	0.3957	85.0	1345600
9.0	23.0	0.2721	622800	0.4995	84.5	1246800
7.0	20.0	0.2453	516300	0.3753	81.5	1376000
5.0	13.0	0.1575	355400	0.2766	82.5	1284900
4.0	13.0	0.1382	173900	0.1401	94.0	1241600
2.0	4.0	0.0506	108500	0.0822	79.0	1320600
1.0	3.5	0.0374	64100	0.0452	93.5	1419100
20.0	51.5	0.6688	936100	0.7338	77.0	1275800
10.0	28.0	0.3146	546500	0.4056	89.0	1347400
9.0	23.0	0.2721	589000	0.4755	84.5	1238800
7.0	19.0	0.2209	411400	0.3113	86.0	1321800
5.0	15.5	0.1684	279200	0.2184	92.0	1278600
4.0	11.5	0.1210	161500	0.1244	95.0	1298500
2.0	5.5	0.0643	133200	0.1044	85.5	1275800
1.0	4.0	0.0414	66700	0.0489	96.5	1365000
20.0	54.5	0.6812	1011600	0.7759	80.0	1303700
10.0	28.5	0.3202	560700	0.4163	89.0	1347100
9.0	29.0	0.2761	654900	0.5261	105.0	1244900
7.0	19.0	0.2275	522300	0.4063	83.5	1285600
5.0	13.0	0.1405	281400	0.2227	92.5	1263900
4.0	12.0	0.1293	218300	0.1528	93.0	1428600
2.0	5.0	0.0591	133500	0.0970	84.5	1375900
1.0	3.5	0.0366	67100	0.0508	95.5	1320800

Table 3.3: Experimental Data for Nicotine-1'-N-oxide (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	60.5	0.7756	869100	0.6708	78.0	1295800
10.0	28.5	0.3149	589400	0.4172	90.5	1412900
9.0	28.5	0.2835	640200	0.5051	100.5	1267600
7.0	19.0	0.2248	511300	0.3980	84.5	1285000
5.0	12.5	0.1351	288200	0.2260	92.5	1275400
4.0	12.0	0.1325	192000	0.1405	90.5	1367200
2.0	6.0	0.0689	143300	0.1148	87.0	1248800
1.0	3.5	0.0366	60200	0.0435	95.5	1386300
20.0	58.5	0.7134	960900	0.7088	82.0	1355700
10.0	27.0	0.3033	541000	0.3809	89.0	1420500
9.0	25.0	0.2551	622700	0.4868	98.0	1279400
7.0	18.5	0.2256	496300	0.3843	82.0	1291500
5.0	13.0	0.1397	323000	0.2462	93.0	1311900
4.0	11.5	0.1292	225500	0.1600	89.0	1410100
2.0	5.0	0.0529	105800	0.0865	94.5	1224000
1.0	3.0	0.0342	38000	0.0268	87.5	1420300
20.0	54.0	0.7346	919300	0.6991	73.5	1315000
10.0	28.0	0.3043	494000	0.3611	92.0	1368300
9.0	25.0	0.2564	648600	0.4764	97.5	1361700
7.0	19.0	0.2345	517600	0.3918	81.0	1321400
5.0	13.0	0.1382	334700	0.2535	94.0	1320600
4.0	10.5	0.1242	227700	0.1618	84.5	1407800
2.0	5.5	0.0597	102200	0.0796	92.0	1284900
1.0	4.0	0.0412	46800	0.0329	97.0	1422600
20.0	58.0	0.6946	1065800	0.8062	83.5	1322000
10.0	28.5	0.3048	541400	0.3987	93.5	1357900
9.0	26.0	0.2708	616900	0.4639	96.0	1330000
7.0	16.0	0.2352	448200	0.3623	68.0	1237300
5.0	14.0	0.1505	336500	0.2555	93.0	1317500
4.0	10.0	0.1183	197400	0.1517	84.5	1302100
2.0	5.5	0.0591	122500	0.0908	93.0	1350200
1.0	3.0	0.0309	50700	0.0346	97.0	1468200

Table 3.3: Experimental Data for Nicotine-1'-N-oxide (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	58.5	0.6763	1076100	0.8354	86.5	1288200
10.0	27.5	0.3021	480400	0.3646	91.0	1317700
9.0	27.5	0.2791	583700	0.4428	98.5	1318200
7.0	16.0	0.2352	455100	0.3758	68.0	1210900
5.0	14.0	0.1521	340900	0.2526	92.0	1349700
4.0	11.5	0.1419	191100	0.1563	81.0	1222900
2.0	6.5	0.0714	120100	0.0904	91.0	1328900
1.0	3.5	0.0355	42600	0.0306	98.5	1391200
20.0	58.0	0.6553	1032700	0.7641	88.5	1351600
10.0	28.5	0.3202	543400	0.3883	89.0	1399400
9.0	27.0	0.2812	612400	0.4585	96.0	1335800
7.0	17.0	0.2165	544400	0.4222	78.5	1289600
5.0	14.0	0.1513	339600	0.2492	92.5	1363100
4.0	11.0	0.1208	175900	0.1287	91.0	1366400
2.0	6.0	0.0794	129100	0.0968	75.5	1334600
1.0	3.5	0.0357	49400	0.0351	98.0	1409400
20.0	60.5	0.6685	1198700	0.8670	90.5	1382500
10.0	28.0	0.3128	560200	0.4098	89.5	1367100
9.0	26.0	0.2751	631000	0.4729	94.5	1334500
7.0	18.5	0.2371	457600	0.3658	78.0	1251100
5.0	14.0	0.1530	340600	0.2624	91.5	1297900
4.0	12.5	0.1420	159800	0.1316	88.0	1215100
2.0	5.5	0.0774	82200	0.0683	71.0	1204100
1.0	3.5	0.0357	50700	0.0374	98.0	1356400
20.0	60.5	0.6540	1194500	0.8321	92.5	1435600
10.0	28.0	0.3163	528000	0.3767	88.5	1401800
9.0	24.5	0.2578	610200	0.4575	95.0	1333700
7.0	18.5	0.2312	505000	0.3889	80.0	1298600
5.0	13.0	0.1428	344800	0.2611	91.0	1320900
4.0	12.0	0.1387	157800	0.1290	86.5	1223200
2.0	5.0	0.0645	83000	0.0700	77.5	1185300
1.0	3.5	0.0364	50500	0.0371	96.0	1364900

TABLE 3.4: Experimental Data for 3' Hydroxycotinine over the concentration range 1-20 µg ml^{-1} (n=12)

X	Y				IS	
Concn. µg ml^{-1}	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	152.5	2.0333	2373100	1.8433	75.0	1287400
10.0	83.5	1.0986	1093000	0.8069	76.0	1354600
9.0	83.0	1.0184	1072300	0.8172	81.5	1312200
7.0	62.5	0.7309	850400	0.6467	85.5	1315100
5.0	40.0	0.5161	543900	0.4458	77.5	1220200
4.0	36.0	0.3934	472700	0.3811	91.5	1240600
2.0	15.5	0.1987	208800	0.1710	78.0	1221100
1.0	8.0	0.0930	87400	0.0633	86.0	1382900
20.0	161.0	2.1184	1950800	1.7262	76.0	1130100
10.0	85.0	1.0000	1140300	0.8474	85.0	1345600
9.0	77.0	0.9112	1038000	0.8325	84.5	1246800
7.0	64.5	0.7914	859100	0.6244	81.5	1376000
5.0	41.0	0.4969	550200	0.4282	82.5	1284900
4.0	39.5	0.4202	461500	0.3717	94.0	1241600
2.0	16.0	0.2025	181500	0.1375	79.0	1320600
1.0	8.0	0.0855	89700	0.0632	93.5	1419100
20.0	148.0	1.9220	2306000	1.8075	77.0	1275800
10.0	86.0	0.9662	1184300	0.8790	89.0	1347400
9.0	78.0	0.9230	1013700	0.8183	84.5	1238800
7.0	63.5	0.7383	822100	0.6220	86.0	1321800
5.0	50.0	0.5434	569500	0.4454	92.0	1278600
4.0	36.0	0.3789	471700	0.3633	95.0	1298500
2.0	16.0	0.1871	195900	0.1536	85.5	1275800
1.0	9.0	0.0932	81600	0.0598	96.5	1365000
20.0	162.0	2.0250	2382700	1.8276	80.0	1303700
10.0	87.6	0.9831	1196800	0.8884	89.0	1347100
9.0	105.5	1.0047	1001500	0.8045	105.0	1244900
7.0	64.0	0.7664	875800	0.6813	83.5	1285600
5.0	45.0	0.4864	609600	0.4824	92.5	1263900
4.0	34.5	0.3709	483100	0.3382	93.0	1428600
2.0	14.5	0.1715	213900	0.1555	84.5	1375900
1.0	8.0	0.0837	105600	0.0800	95.5	1320800

TABLE 3.4: Experimental Data for 3' Hydroxycotinine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	166.5	2.1346	2415200	1.8639	78.0	1295800
10.0	90.0	0.9944	1261700	0.8930	90.5	1412900
9.0	99.0	0.9850	1022300	0.8065	100.5	1267600
7.0	64.0	0.7573	864600	0.6728	84.5	1285000
5.0	46.0	0.4972	605300	0.4746	92.5	1275400
4.0	35.0	0.3867	455300	0.3331	90.5	1367200
2.0	16.5	0.1896	158700	0.1271	87.0	1248800
1.0	8.0	0.0837	93000	0.0671	95.5	1386300
20.0	168.5	2.0548	2384000	1.7585	82.0	1355700
10.0	92.5	1.0393	1199500	0.8444	89.0	1420500
9.0	90.0	0.9183	1081500	0.8453	98.0	1279400
7.0	62.0	0.7560	856700	0.6634	82.0	1291500
5.0	45.0	0.4838	589000	0.4490	93.0	1311900
4.0	35.0	0.3932	466800	0.3310	89.0	1410100
2.0	16.5	0.1746	195500	0.1598	94.5	1224000
1.0	7.5	0.0857	116000	0.0817	87.5	1420300
20.0	142.5	1.9387	2200300	1.6732	73.5	1315000
10.0	91.0	0.9891	1131200	0.8267	92.0	1368300
9.0	85.0	0.8717	1087400	0.7986	97.5	1361700
7.0	60.0	0.7407	860200	0.6510	81.0	1321400
5.0	44.0	0.4680	573700	0.4344	94.0	1320600
4.0	33.0	0.3905	491500	0.3492	84.5	1407800
2.0	17.0	0.1847	186800	0.1454	92.0	1284900
1.0	8.5	0.0876	87900	0.0618	97.0	1422600
20.0	167.5	2.0059	2285400	1.7287	83.5	1322000
10.0	93.5	1.0000	1148100	0.8455	93.5	1357900
9.0	84.0	0.8750	1053500	0.7921	96.0	1330000
7.0	52.0	0.7647	812500	0.6567	68.0	1237300
5.0	45.5	0.4892	580300	0.4405	93.0	1317500
4.0	31.5	0.3727	466600	0.3584	84.5	1302100
2.0	16.0	0.1720	214500	0.1589	93.0	1350200
1.0	9.0	0.0927	85500	0.0583	97.0	1468200

TABLE 3.4: Experimental Data for 3' Hydroxycotinine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Height (counts)
20.0	174.0	2.0115	2366700	1.8372	86.5	1288200
10.0	92.0	1.0109	1102800	0.8369	91.0	1317700
9.0	85.0	0.8629	1006300	0.7634	98.5	1318200
7.0	52.0	0.7647	786200	0.6493	68.0	1210900
5.0	45.5	0.4945	594100	0.4402	92.0	1349700
4.0	36.5	0.4506	438500	0.3586	81.0	1222900
2.0	17.5	0.1923	225900	0.1700	91.0	1328900
1.0	8.5	0.0862	79200	0.0570	98.5	1391200
20.0	177.0	2.0000	2459400	1.8196	88.5	1351600
10.0	90.5	1.0168	1202600	0.8594	89.0	1399400
9.0	86.0	0.8958	1039000	0.7778	96.0	1335800
7.0	54.5	0.6942	788100	0.6112	78.5	1289600
5.0	44.0	0.4756	594500	0.4362	92.5	1363100
4.0	34.0	0.3736	494200	0.3617	91.0	1366400
2.0	16.0	0.2119	189400	0.1419	75.5	1334600
1.0	8.5	0.0867	89300	0.0634	98.0	1409400
20.0	181.5	2.0055	2607500	1.8861	90.5	1382500
10.0	90.5	1.0111	1215800	0.8893	89.5	1367100
9.0	82.5	0.8730	1043900	0.7822	94.5	1334500
7.0	61.0	0.7820	818900	0.6546	78.0	1251100
5.0	41.0	0.4480	540900	0.4168	91.5	1297900
4.0	36.5	0.4147	423400	0.3486	88.0	1215100
2.0	15.0	0.2112	168300	0.1398	71.0	1204100
1.0	9.5	0.0969	89700	0.0662	98.0	1356400
20.0	179.0	1.9351	2607200	1.8161	92.5	1435600
10.0	89.5	1.0112	1210100	0.8632	88.5	1401800
9.0	85.0	0.8947	1055400	0.7913	95.0	1333700
7.0	58.0	0.7250	835200	0.6432	80.0	1298600
5.0	45.0	0.4945	611000	0.4626	91.0	1320900
4.0	37.0	0.4277	447500	0.3659	86.5	1223200
2.0	13.4	0.1729	168700	0.1424	77.5	1185300
1.0	9.0	0.0937	113000	0.0828	96.0	1364900

TABLE 3.5: Experimental Data for Cotinine over the concentration range 1-20 $\mu g\ ml^{-1}$ (n=12)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	145.5	1.9400	2782500	2.1613	75.0	1287400
10.0	78.0	1.0263	1446700	1.0680	76.0	1354600
9.0	79.0	0.9693	1267600	0.9660	81.5	1312200
7.0	64.0	0.7485	963000	0.7323	85.5	1315100
5.0	39.5	0.5096	624800	0.5121	77.5	1220200
4.0	41.0	0.4480	529300	0.4267	91.5	1240600
2.0	16.0	0.2051	245400	0.2010	78.0	1221100
1.0	8.0	0.0930	100700	0.0729	86.0	1382900
20.0	148.0	1.9473	2421200	2.1425	76.0	1130100
10.0	79.5	0.9352	1439900	1.0701	85.0	1345600
9.0	76.0	0.8994	1165800	0.9350	84.5	1246800
7.0	61.0	0.7484	999200	0.7262	81.5	1376000
5.0	42.0	0.5090	661700	0.5150	82.5	1284900
4.0	41.5	0.4414	535600	0.4314	94.0	1241600
2.0	17.5	0.2215	264800	0.2006	79.0	1320600
1.0	8.0	0.0855	147800	0.1042	93.5	1419100
20.0	145.5	1.8896	2766300	2.1683	77.0	1275800
10.0	84.0	0.9438	1458600	1.0825	89.0	1347400
9.0	76.0	0.8994	1157900	0.9347	84.5	1238800
7.0	64.0	0.7441	995000	0.7528	86.0	1321800
5.0	47.0	0.5108	698200	0.5461	92.0	1278600
4.0	42.5	0.4473	561900	0.4327	95.0	1298500
2.0	17.0	0.1988	249700	0.1957	85.5	1275800
1.0	8.5	0.0880	142900	0.1047	96.5	1365000
20.0	158.0	1.9750	2877900	2.2075	80.0	1303700
10.0	86.5	0.9719	1455000	1.0801	89.0	1347100
9.0	101.0	0.9619	1227000	0.9856	105.0	1244900
7.0	63.0	0.7544	969200	0.7539	83.5	1285600
5.0	46.5	0.5027	687800	0.5442	92.5	1263900
4.0	40.5	0.4354	585400	0.4098	93.0	1428600
2.0	17.5	0.2071	270400	0.1965	84.5	1375900
1.0	9.0	0.0942	149900	0.1136	95.5	1320800

TABLE 3.5: Experimental Data for Cotinine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	151.5	1.9423	2874600	2.2184	78.0	1295800
10.0	87.5	0.9668	1519700	1.0756	90.5	1412900
9.0	93.5	0.9303	1204300	0.9501	100.5	1267600
7.0	63.0	0.7455	987200	0.7683	84.5	1285000
5.0	46.5	0.5027	669800	0.5252	92.5	1275400
4.0	41.0	0.4530	595900	0.4359	90.5	1367200
2.0	17.0	0.1954	241700	0.1936	87.0	1248800
1.0	9.5	0.0994	119600	0.0863	95.5	1386300
20.0	154.5	1.8841	2937200	2.1666	82.0	1355700
10.0	85.0	0.9550	1520500	1.0704	89.0	1420500
9.0	88.0	0.8979	1245100	0.9732	98.0	1279400
7.0	61.5	0.7500	993700	0.7694	82.0	1291500
5.0	46.5	0.5000	676700	0.5158	93.0	1311900
4.0	39.0	0.4382	551300	0.3910	89.0	1410100
2.0	18.5	0.1957	245200	0.2004	94.5	1224000
1.0	9.5	0.1085	128600	0.0906	87.5	1420300
20.0	143.5	1.9523	2873800	2.1854	73.5	1315000
10.0	89.5	0.9728	1416700	1.0354	92.0	1368300
9.0	85.0	0.8717	1244400	0.9139	97.5	1361700
7.0	59.5	0.7345	976900	0.7393	81.0	1321400
5.0	46.0	0.4893	685700	0.5193	94.0	1320600
4.0	38.5	0.4556	576600	0.4096	84.5	1407800
2.0	17.5	0.1902	257100	0.2002	92.0	1284900
1.0	9.0	0.0927	145500	0.1023	97.0	1422600
20.0	162.0	1.9401	2891000	2.1869	83.5	1322000
10.0	90.5	0.9679	1478700	1.0890	93.5	1357900
9.0	88.0	0.9166	1318700	0.9915	96.0	1330000
7.0	51.0	0.7500	956400	0.7730	68.0	1237300
5.0	46.0	0.4946	706000	0.5359	93.0	1317500
4.0	33.0	0.3905	572700	0.4399	84.5	1302100
2.0	18.0	0.1935	279300	0.2069	93.0	1350200
1.0	9.5	0.0979	115500	0.0787	97.0	1468200

TABLE 3.5: Experimental Data for Cotinine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	165.0	1.9075	2851400	2.2135	86.5	1288200
10.0	90.0	0.9890	1384500	1.0507	91.0	1317700
9.0	86.5	0.8781	1243000	0.9430	98.5	1318200
7.0	51.5	0.7573	882600	0.7289	68.0	1210900
5.0	47.0	0.5108	621500	0.4605	92.0	1349700
4.0	32.5	0.4012	497300	0.4067	81.0	1222900
2.0	18.0	0.1978	285100	0.2146	91.0	1328900
1.0	9.5	0.0964	165900	0.1193	98.5	1391200
20.0	167.5	1.8926	3008600	2.2260	88.5	1351600
10.0	88.0	0.9887	1519300	1.0857	89.0	1399400
9.0	86.0	0.8958	1298600	0.9721	96.0	1335800
7.0	54.5	0.6942	956500	0.7417	78.5	1289600
5.0	45.5	0.4918	675400	0.4956	92.5	1363100
4.0	35.5	0.3901	556700	0.4075	91.0	1366400
2.0	15.0	0.1986	257500	0.1929	75.5	1334600
1.0	9.5	0.0969	120700	0.0857	98.0	1409400
20.0	173.0	1.9116	3082300	2.2295	90.5	1382500
10.0	88.0	0.9832	1520000	1.1119	89.5	1367100
9.0	83.5	0.8835	1256200	0.9413	94.5	1334500
7.0	57.5	0.7371	943900	0.7545	78.0	1251100
5.0	45.5	0.4972	709200	0.5465	91.5	1297900
4.0	34.0	0.3863	532700	0.4384	88.0	1215100
2.0	14.5	0.2042	241300	0.2004	71.0	1204100
1.0	9.5	0.0969	139700	0.1030	98.0	1356400
20.0	174.0	1.8810	3173900	2.2108	92.5	1435600
10.0	87.5	0.9887	1544700	1.1019	88.5	1401800
9.0	83.5	0.8789	1262700	0.9468	95.0	1333700
7.0	58.0	0.7250	970200	0.7471	80.0	1298600
5.0	45.5	0.5000	703000	0.5322	91.0	1320900
4.0	36.0	0.4161	529000	0.4326	86.5	1223200
2.0	16.0	0.2064	235100	0.1984	77.5	1185300
1.0	9.0	0.0937	124800	0.0914	96.0	1364900

TABLE 3.6: Experimental Data for Nicotine over the concentration range 1-20 μg ml^{-1} (n=12)

X	Y				IS	
Concn. μg ml^{-1}	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	108.0	1.4400	3026900	2.3512	75.0	1287400
10.0	58.5	0.7697	1438000	1.0616	76.0	1354600
9.0	58.5	0.7177	1289700	0.9828	81.5	1312200
7.0	46.0	0.5380	979300	0.7447	85.5	1315100
5.0	30.0	0.3870	624800	0.5121	77.5	1220200
4.0	23.5	0.2568	467600	0.3770	91.5	1240600
2.0	12.5	0.1602	239800	0.1964	78.0	1221100
1.0	7.0	0.0813	87500	0.0633	86.0	1382900
20.0	108.5	1.4276	2572600	2.2764	76.0	1130100
10.0	67.0	0.7882	1447800	1.0760	85.0	1345600
9.0	59.5	0.7041	1281000	1.027	84.5	1246800
7.0	45.5	0.5582	995900	0.7238	81.5	1376000
5.0	32.0	0.3878	615100	0.4788	82.5	1284900
4.0	23.0	0.2446	448500	0.3613	94.0	1241600
2.0	12.0	0.1518	239700	0.1816	79.0	1320600
1.0	7.0	0.0748	118500	0.0835	93.5	1419100
20.0	105.0	1.3636	2923600	2.2916	77.0	1275800
10.0	66.5	0.7471	1429900	1.0612	89.0	1347400
9.0	58.5	0.6923	1214700	0.9806	84.5	1238800
7.0	46.5	0.5406	978000	0.7400	86.0	1321800
5.0	33.0	0.3586	620000	0.4849	92.0	1278600
4.0	24.5	0.2578	468000	0.3604	95.0	1298500
2.0	13.0	0.1520	226700	0.1777	85.5	1275800
1.0	7.5	0.0777	101800	0.0746	96.5	1365000
20.0	112.5	1.4062	3180100	2.4393	80.0	1303700
10.0	67.5	0.7584	1441700	1.0702	89.0	1347100
9.0	64.0	0.6095	1239200	0.9954	105.0	1244900
7.0	46.0	0.5508	1081900	0.8416	83.5	1285600
5.0	33.5	0.3621	610900	0.4834	92.5	1263900
4.0	24.5	0.2634	439100	0.3074	93.0	1428600
2.0	14.0	0.1656	255700	0.1859	84.5	1375900
1.0	7.0	0.0732	64900	0.0492	95.5	1320800

TABLE 3.6: Experimental Data for Nicotine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	108.0	1.3846	3093900	2.3876	78.0	1295800
10.0	66.5	0.7348	1441600	1.0203	90.5	1412900
9.0	66.5	0.6616	1328000	1.0476	100.5	1267600
7.0	46.5	0.5502	958700	0.7461	84.5	1285000
5.0	35.0	0.3783	656500	0.5148	92.5	1275400
4.0	22.0	0.2430	457100	0.3344	90.5	1367200
2.0	14.0	0.1609	231500	0.1854	87.0	1248800
1.0	7.5	0.0785	88300	0.0637	95.5	1386300
20.0	112.0	1.3658	3338500	2.4626	82.0	1355700
10.0	63.5	0.7134	1435200	1.0104	89.0	1420500
9.0	65.5	0.6683	1319700	1.0315	98.0	1279400
7.0	46.0	0.5609	1005000	0.7782	82.0	1291500
5.0	36.0	0.3870	675400	0.5149	93.0	1311900
4.0	21.5	0.2415	434700	0.3083	89.0	1410100
2.0	15.5	0.1640	233800	0.1910	94.5	1224000
1.0	6.5	0.0742	136900	0.0964	87.5	1420300
20.0	102.0	1.3877	3417500	2.5989	73.5	1315000
10.0	65.0	0.7065	1423600	1.0404	92.0	1368300
9.0	65.0	0.6666	1317000	0.9672	97.5	1361700
7.0	47.0	0.5802	1002800	0.7589	81.0	1321400
5.0	36.5	0.3882	665600	0.5040	94.0	1320600
4.0	21.5	0.2544	425000	0.3019	84.5	1407800
2.0	14.5	0.1576	256300	0.1995	92.0	1284900
1.0	7.0	0.0721	106500	0.0749	97.0	1422600
20.0	112.5	1.3554	337370 0	2.5520	83.5	1322000
10.0	67.0	0.7165	144110 0	1.0613	93.5	1357900
9.0	67.5	0.7031	133430 0	1.0032	96.0	1330000
7.0	38.5	0.5661	93030 0	0.7519	68.0	1237300
5.0	36.5	0.3924	72570 0	0.5509	93.0	1317500
4.0	19.0	0.2248	35220 0	0.2705	84.5	1302100
2.0	15.0	0.1612	27920 0	0.2068	93.0	1350200
1.0	7.5	0.0773	10770 0	0.0734	97.0	1468200

TABLE 3.6: Experimental Data for Nicotine (continued)

X	Y				IS	
Concn. $\mu g\ ml^{-1}$	Peak Height (mm)	Peak Height Ratio	Peak Area (counts)	Peak Area Ratio	Peak Height (mm)	Peak Area (counts)
20.0	117.0	1.3526	2972600	2.3076	86.5	1288200
10.0	65.5	0.7197	1398700	1.0615	91.0	1317700
9.0	65.0	0.6598	1213100	0.9203	98.5	1318200
7.0	38.5	0.5661	897800	0.7415	68.0	1210900
5.0	36.0	0.3913	676600	0.5014	92.0	1349700
4.0	17.5	0.2160	424700	0.3473	81.0	1222900
2.0	15.5	0.1703	233500	0.1757	91.0	1328900
1.0	8.0	0.0812	91000	0.0654	98.5	1391200
20.0	121.0	1.3672	2998200	2.2183	88.5	1351600
10.0	65.0	0.7303	1452000	1.0376	89.0	1399400
9.0	64.5	0.6718	1291200	0.9666	96.0	1335800
7.0	46.0	0.5859	1004200	0.7787	78.5	1289600
5.0	36.0	0.3891	678300	0.4976	92.5	1363100
4.0	20.0	0.2197	429800	0.3146	91.0	1366400
2.0	13.0	0.1721	252900	0.1895	75.5	1334600
1.0	7.0	0.0714	79700	0.0566	98.0	1409400
20.0	125.0	1.3812	3203600	2.3172	90.5	1382500
10.0	65.0	0.7262	1458400	1.0668	89.5	1367100
9.0	64.5	0.6825	1351900	1.0130	94.5	1334500
7.0	42.0	0.5384	889000	0.7106	78.0	1251100
5.0	35.5	0.3879	686000	0.5286	91.5	1297900
4.0	19.0	0.2159	363100	0.2989	88.0	1215100
2.0	12.5	0.1760	210800	0.1751	71.0	1204100
1.0	6.5	0.0663	105800	0.0780	98.0	1356400
20.0	126.0	1.3621	3436000	2.3934	92.5	1435600
10.0	63.0	0.7118	1515000	1.0808	88.5	1401800
9.0	64.5	0.6789	1388300	1.0409	95.0	1333700
7.0	45.0	0.5625	1006600	0.7751	80.0	1298600
5.0	35.5	0.3901	703400	0.5326	91.0	1320900
4.0	18.5	0.2138	412900	0.3376	86.5	1223200
2.0	12.0	0.1548	236500	0.1996	77.5	1185300
1.0	6.5	0.0677	112400	0.0824	96.0	1364900

TABLE 3.7: Reproducibility Data (Expressed as Relative Standard Deviations) for each analyte over a range of concentrations using different measurement parameters and collectively for each measurement parameter

Measurement Parameter	Statistic (%)	Nicotine-1'-N-oxide	3' Hydroxy-cotinine	Cotinine	Internal Standard	Nicotine	Mean RSD for all analyte peaks
Peak Height	Range	2.7 - 11.7	3.5 - 9.4	4.6 - 9.4	4.3 - 9.2	3.8 - 11.3	6.9
	Mean ± SD	7.4 ± 2.6	6.8 ± 1.7	7.0 ± 1.6	6.5 ± 1.8	7.0 ± 2.4	
Peak Height Ratio	Range	4.3 - 14.0	3.3 - 7.8	1.5 - 6.3	-	2.0 - 7.8	4.9
	Mean ± SD	6.8 ± 3.2	5.0 ± 1.6	3.5 ± 1.9	-	4.3 ± 2.0	
Peak Area	Range	4.0 - 21.2	2.7 - 12.8	3.3 - 13.6	2.3 - 6.3	1.9 - 19.1	7.0
	Mean ± SD	11.3 ± 6.2	6.3 ± 3.7	5.8 ± 3.4	3.9 ± 1.5	7.5 ± 5.2	
Peak Area Ratio	Range	5.1 - 22.5	2.9 - 13.7	1.3 - 14.6	-	2.1 - 18.0	6.7
	Mean ± SD	10.5 ± 6.1	5.5 ± 3.8	4.2 ± 4.3	-	6.5 ± 5.1	

were found to be equivalent in terms of reproducibility with RSDs of 6.9% and 7.0% respectively.

The inclusion of an IS results in an improvement in the RSD averaged over the concentration range, for each analyte and in the mean RSD averaged over all concentrations and standards for each particular method of quantitation. The best method of quantitation, in terms of between-run precision, was peak height ratio (RSD = 4.9%), followed by peak area ratio (RSD = 6.7%).

Errors arise in the experiment due to the use of gradient elution which causes random changes in the baseline over a particular run, and uncertainty in marking start and end-points of the peaks for integration. Errors also occur in the preparation of the standard solutions and the mobile phase, and as the HPLC system was not thermostated, changes in the ambient temperature on the day cause variation in the chromatograms obtained; this is more important when the experiment is carried out over a period of weeks than when only one day is being considered. Over a period of time, the standards show some deterioration and also the column performance decreases with age.

3.4 The Calibration Curve [202]

The analyses to be carried out on urine samples as the conclusion of this method development will be based on graphical methods, where a calibration curve is constructed from a series of measurements on a group of standards of known concentration over the range of interest. The graph takes the form of a highly precise measurement, such as concentration, on the x-axis with observed values of a less precise measurement such as peak area (peak area ratio, peak height or peak height ratio) on the y-axis.

All measurements are subject to errors and hence it is necessary to assess the errors involved in constructing the calibration curve, especially as the calibration line will be used to find the x-value of a sample when the y-value has been measured.

Firstly the 'best' straight line through the calibration graph points must be calculated. This is done using the method of least squares, so called because it minimizes the sum of the squares of the residuals.

The line of regression is given by:

$$Y = a + bX$$

where a = the intercept on the y-axis

and b = the gradient of the regression line.

An example calculation is given for the nicotine-1'-N-oxide with peak height as the measurement parameter at the end of this section.

A confidence region, about the entire line, the Working-Hotelling region, first proposed in 1929, is given by:

$$Y = a + bX \pm \left[2F_{2,\nu,\alpha} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2}\right) s^2 \right]^{\frac{1}{2}}$$

where F = Snedecor's F statistic

ν = degrees of freedom = n-2

α = probability level for the desired confidence interval $100(1-\alpha)\%$
e.g. $\alpha = 0.05$ for 95% confidence interval

n = number of observations

$\Sigma x^2 = \Sigma(X-\bar{X})^2$

and s^2 = variance

In 1953, Scheffé showed that the Working-Hotelling confidence bands about the calibration line give an estimate of the error inherent in the determination which is valid whatever the setting of x. The 95% confidence region for the entire line is illustrated along with the fitted line in figure 3.3(a). A complete calculation is also given, at the end of this section, using the data recorded for nicotine-1'-N-oxide quantitated by peak height measurements.

Once a fitted line is obtained, it is often required to be used over and over again for all future values of the abscissa variable x (concentration) for estimating the associated future observations y* (peak height, peak area etc.).

The most practical method for producing confidence limits for the repeated use of the fitted line for all future observations employs the Working-Hotelling confidence region combined with tolerance limits for the observations. The tolerance limits give bounds within which a certain proportion P of the future observations will be found. The tolerance bounds calculated contain P = 0.90 or 90% of the future observations y with 95% confidence, for any and all values of the abscissa variable x. These tolerance bounds are given by:

$$Y = a + bX \pm \{A + z_p B\}$$

where $A = \left[2F_{2,\nu,\alpha/2} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2}\right) s^2 \right]^{\frac{1}{2}}$

$$B = \left[\nu s^2 / \chi^2_{\nu,1-\alpha/2} \right]^{\frac{1}{2}}$$

and z_p = the normal deviate.

(For $100(1-\alpha)\%$ overall confidence, the critical values of both F and χ^2 have been set at $\alpha/2$). The tolerance bounds are also illustrated in figure 3.3(a) and a complete example calculation has been included using the data recorded for the nicotine-1'-N-oxide standard quantitated by peak height measurements.

For each of the four standard components and each method of quantitation, the line of 'best' fit, Working-Hotelling 95% confidence bands and 95% confidence bounds for 90% of future observations have been calculated and the data summarized in tables 3.9 to 3.12. The graphs showing the fitted model with the corresponding Working-Hotelling 90% confidence region and the 95% confidence bounds for 90% of future observations are presented in figures 3.3 to 3.6.

Construction of the Calibration Curve of Nicotine-1'-N-oxide from Peak Height Measurements with Fitted Regression Line, Working-Hotelling 95% Confidence Region and 95% Confidence Bounds for 90% of Future Observations

TABLE 3.8: Calibration Data

X = concentration in $\mu g\ ml^{-1}$

Y = peak height in mm.

Concn. X	Peak Height Y	$(X-\bar{X})=x$	$(Y-\bar{Y})=y$	x^2	y^2	xy
20.0	56.8	12.75	36.45	162.5625	1328.6025	464.7375
10.0	27.9	2.75	7.55	7.5625	57.0025	20.7625
9.0	25.7	1.75	5.35	3.0625	28.6225	9.3625
7.0	18.5	-0.25	-1.85	0.0625	3.4225	0.4625
5.0	13.4	-2.25	-6.95	5.0625	48.3025	15.6375
4.0	11.6	-3.25	-8.75	10.5625	76.5625	28.4375
2.0	5.4	-5.25	-14.95	27.5625	223.5025	78.4875
1.0	3.5	-6.25	-16.85	39.0625	283.9225	105.3125

$\Sigma X = 58$ $\Sigma Y = 162.8$ $\Sigma x^2 = 255.5$

$\bar{X} = 7.25$ $\bar{Y} = 20.35$ $\Sigma y^2 = 2049.94$

$\Sigma xy = 723.2$

$$Y = a + bX$$

$$\text{Gradient, } b = \frac{\Sigma xy}{\Sigma x^2} = \frac{723.2}{255.5} = 2.830528376$$

$$\text{Intercept, } a = \bar{Y} - b\bar{X} = 20.35 - (2.830528376 \times 7.25)$$

$$= -0.171330723$$

$\therefore$ Regression line is given by

$$\hat{Y} = -0.171330723 + 2.830528376\ X$$

$$\text{Variance, } s^2 = \frac{\Sigma y^2 - b\Sigma xy}{n-2}$$

$$= \frac{2049.94 - (2.830528376 \times 723.2)}{6}$$

$$= 0.483646416$$

$$s_{YX} = 0.695446918$$

(Additional decimal places are used in the computations to keep rounding errors to a minimum.)

Working-Hotelling 95% Confidence Region

$$Y = a + bX \pm \left[2F_{2,\nu,\alpha} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2}\right) s^2 \right]^{\frac{1}{2}}$$

Since $F_{2,\nu,\alpha} = F_{2,6,0.05} = 5.143$ (Tables)

Then

$$Y' = -0.171330723 + 2.830528376\ X' \pm \left[2 \times 5.143 \left(\frac{1}{8} + \frac{(X' - 7.25)^2}{255.5}\right) 0.483646416 \right]^{\frac{1}{2}}$$

$$= -0.171330723 + 2.830528376\ X' \pm \left[4.974787035 \left(0.125 + \frac{(X'-7.25)^2}{255.5}\right) \right]^{\frac{1}{2}}$$

$\therefore$ For $X_1 = 20.0$, $Y_1 = 56.44 \pm 1.95$

$= 58.39$ or 54.49

For $X_2 = 10.0$, $Y_2 = 28.13 \pm 0.88$

$= 29.01$ or 27.25

For $X_3 = 9.0$, $Y_3 = 25.30 \pm 0.83$

$= 26.13$ or 24.47

For $X_4 = 7.0$, $Y_4 = 19.64 \pm 0.79$

$= 20.43$ or 18.85

For $X_5 = 5.0$, $Y_5 = 13.98 \pm 0.85$

$= 14.83$ or 13.13

For $X_6 = 4.0$, $Y_6 = 11.15 \pm 0.91$

$= 12.06$ or 10.24

For $X_7 = 2.0$, $Y_7 = 5.49 \pm 1.08$

$= 6.57$ or 4.41

For $X_8 = 1.0$, $Y_8 = 2.66 \pm 1.18$

$= 3.84$ or 1.48

<u>95% Confidence Bounds for 90% of Future Observations</u>

$$Y = a + bX \pm (A + z_p B)$$

where $A = \left[2F_{2,\nu,\alpha/2} \left(\frac{1}{n} + \frac{(X-\bar{X})^2}{\Sigma x^2}\right) s^2 \right]^{\frac{1}{2}}$

and $B = \left[\nu s^2 / \chi^2_{\nu,1-\alpha/2} \right]^{\frac{1}{2}}$

Since $z_p = 1.29$ when $P = 0.90$,

$F_{2,6,0.025} = 7.260$ and

$\chi^2_{6,0.975} = 1.24$ (Tables)

Then

$$Y = -0.171330723 + 2.830528376\ X \pm \left(\left[2 \times 7.26 \left(0.125 + \frac{(X-7.25)^2}{255.5} \right) \times 0.483646416 \right]^{\frac{1}{2}} + \left[1.29 \left(\frac{6 \times 0.483646416}{1.24} \right)^{\frac{1}{2}} \right] \right)$$

$$Y = -0.171330723 + 2.830528376\ X \pm \left(\left[7.02254596 \left(0.125 + \frac{(X-7.25)^2}{255.5} \right) \right]^{\frac{1}{2}} + 1.973415249 \right)$$

For $X_1 = 20.0$, $Y_1 = 56.44 \pm 4.29$
$= 60.73$ or 52.15

For $X_2 = 10.0$, $Y_2 = 28.13 \pm 3.02$
$= 31.15$ or 25.11

For $X_3 = 9.0$, $Y_3 = 25.30 \pm 2.95$
$= 28.25$ or 22.35

For $X_4 = 7.0$, $Y_4 = 19.64 \pm 2.91$
$= 22.55$ or 16.73

For $X_5 = 5.0$, $Y_5 = 13.98 \pm 2.98$
$= 16.96$ or 11.00

For $X_6 = 4.0$, $Y_6 = 11.15 \pm 3.05$
$= 14.20$ or 8.10

For $X_7 = 2.0$, $Y_7 = 5.49 \pm 3.25$
$= 8.74$ or 2.24

For $X_8 = 1.0$, $Y_8 = 2.66 \pm 3.37$
$= 6.03$ or -0.71

Figure 3.3(a): Calibration Curve for Nicotine-1'-N-oxide by Peak Height showing the fitted line with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations Y

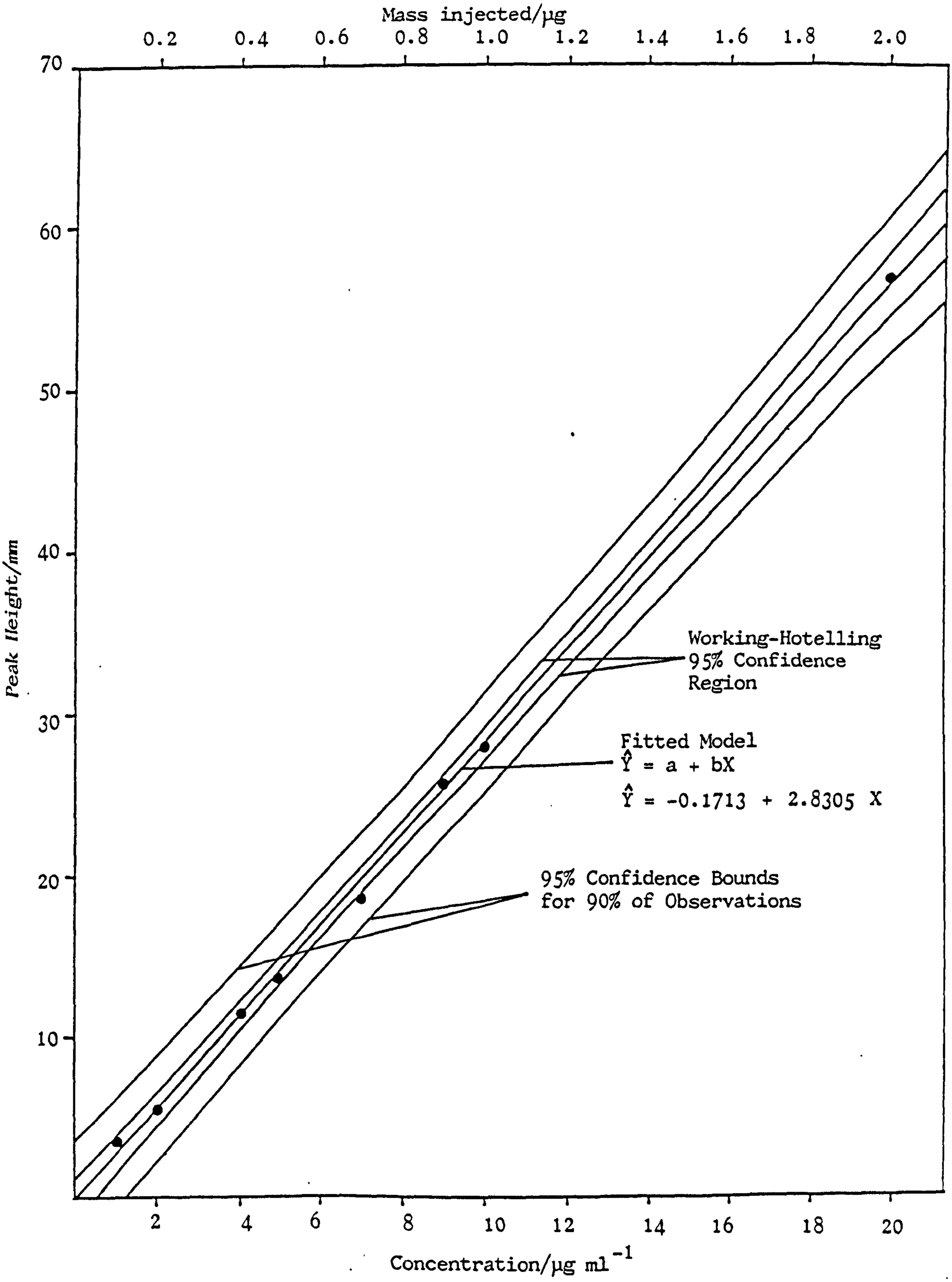


TABLE 3.9: Summary of Calibration Data for Nicotine-1'-N-oxide
(X = concentration in $\mu g\ ml^{-1}$, Y = (a) peak height in mm, (b) peak height ratio, (c) peak area in counts x 10^{-6} and (d) peak area ratio)

(a) $\hat{Y} = -0.1713 + 2.8305\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	56.8	56.4392	58.39	or	54.49	60.73	or	52.15
10.0	27.9	28.1339	29.01	or	27.25	31.15	or	25.11
9.0	25.7	25.3034	26.13	or	24.47	28.25	or	22.35
7.0	18.5	19.6423	20.43	or	18.85	22.55	or	16.73
5.0	13.4	13.9813	14.83	or	13.13	16.96	or	11.00
4.0	11.6	11.1507	12.06	or	10.24	14.20	or	8.10
2.0	5.4	5.4897	6.57	or	4.41	8.74	or	2.24
1.0	3.5	2.6591	3.84	or	1.48	6.03	or	-0.71

(b) $\hat{Y} = -0.0130 + 0.0344\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	0.6944	0.6767	0.73	or	0.63	0.78	or	0.58
10.0	0.3157	0.3318	0.35	or	0.31	0.40	or	0.26
9.0	0.2729	0.2973	0.32	or	0.28	0.37	or	0.23
7.0	0.2322	0.2283	0.25	or	0.21	0.30	or	0.16
5.0	0.1487	0.1593	0.18	or	0.14	0.23	or	0.09
4.0	0.1302	0.1248	0.14	or	0.10	0.19	or	0.05
2.0	0.0649	0.0559	0.09	or	0.03	0.14	or	-0.02
1.0	0.0369	0.0214	0.05	or	-0.01	0.01	or	-0.06

(c) $\hat{Y} = 44023.9276 + 51579.8375\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	1.0203	1.0756	1.28	or	0.88	1.52	or	0.63
10.0	0.5346	0.5598	0.65	or	0.47	0.87	or	0.25
9.0	0.6174	0.5082	0.59	or	0.42	0.81	or	0.20
7.0	0.4922	0.4050	0.49	or	0.32	0.70	or	0.10
5.0	0.3271	0.3019	0.39	or	0.21	0.61	or	-0.01
4.0	0.1882	0.2503	0.34	or	0.16	0.56	or	-0.06
2.0	0.1119	0.1471	0.26	or	0.04	0.48	or	-0.19
1.0	0.0517	0.0956	0.22	or	-0.03	0.44	or	-0.25

(d) $\hat{Y} = 0.0341 + 0.0391\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	0.7780	0.8174	0.98	or	0.65	1.18	or	0.46
10.0	0.3902	0.4257	0.50	or	0.35	0.68	or	0.17
9.0	0.4754	0.3866	0.46	or	0.32	0.64	or	0.14
7.0	0.3815	0.3082	0.38	or	0.24	0.55	or	0.06
5.0	0.2517	0.2299	0.30	or	0.16	0.48	or	-0.02
4.0	0.1433	0.1907	0.27	or	0.11	0.45	or	-0.07
2.0	0.0872	0.1124	0.20	or	0.02	0.39	or	-0.16
1.0	0.0373	0.0733	0.17	or	-0.03	0.36	or	-0.21

TABLE 3.10: Summary of Calibration Data for 3' Hydroxycotinine
(X = concentration in $\mu g\ ml^{-1}$, Y = (a) peak height in mm, (b) peak height ratio, (c) peak area in counts x 10^{-6} and (d) peak area ratio)

(a) $\hat{Y} = 2.5092 + 8.3573\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	165.0	169.6560	182.77	or	156.55	198.53	or	140.79
10.0	89.3	86.0826	91.99	or	80.17	106.39	or	65.77
9.0	86.7	77.7253	82.40	or	73.04	97.62	or	57.82
7.0	59.8	61.0106	66.33	or	55.69	80.62	or	41.40
5.0	44.3	44.2959	50.02	or	38.58	64.38	or	24.22
4.0	35.4	35.9386	42.07	or	29.81	56.51	or	15.37
2.0	15.8	19.2239	26.47	or	11.97	41.13	or	-2.69
1.0	8.5	10.8666	18.79	or	2.95	33.57	or	-11.83

(b) $\hat{Y}\ -3.3878 \times 10^{-3} + 0.1015\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	2.0154	2.0277	2.09	or	1.97	2.16	or	1.90
10.0	1.0101	1.0121	1.04	or	0.98	1.10	or	0.92
9.0	0.9195	0.9106	0.93	or	0.89	1.00	or	0.82
7.0	0.7510	0.7075	0.73	or	0.69	0.80	or	0.62
5.0	0.4912	0.5043	0.52	or	0.48	0.59	or	0.41
4.0	0.3978	0.4028	0.43	or	0.37	0.49	or	0.31
2.0	0.1891	0.1997	0.23	or	0.17	0.30	or	0.10
1.0	0.0891	0.0981	0.14	or	0.06	0.20	or	0.00

(c) $\hat{Y}\ -23719.4784 + 119554.3591\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	2.3615	2.3673	2.41	or	2.33	2.46	or	2.28
10.0	1.1738	1.1718	1.19	or	1.15	1.24	or	1.11
9.0	1.0429	1.0522	1.07	or	1.04	1.12	or	0.99
7.0	0.8358	0.8131	0.83	or	0.80	0.88	or	0.75
5.0	0.5802	0.5740	0.59	or	0.56	0.64	or	0.51
4.0	0.4644	0.4544	0.47	or	0.44	0.52	or	0.39
2.0	0.1923	0.2153	0.24	or	0.19	0.28	or	0.15
1.0	0.0932	0.0958	0.12	or	0.07	0.17	or	0.02

(d) $\hat{Y} = -0.0168 + 0.0906\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	1.7990	1.7968	1.86	or	1.74	1.92	or	1.68
10.0	0.8567	0.8899	0.92	or	0.86	0.98	or	0.80
9.0	0.8025	0.7992	0.82	or	0.78	0.89	or	0.71
7.0	0.6480	0.6179	0.64	or	0.60	0.70	or	0.54
5.0	0.4463	0.4365	0.46	or	0.42	0.53	or	0.35
4.0	0.3551	0.3458	0.38	or	0.32	0.44	or	0.26
2.0	0.1502	0.1645	0.19	or	0.13	0.26	or	0.06
1.0	0.0670	0.0738	0.10	or	0.04	0.17	or	-0.03

TABLE 3.11: Summary of Calibration Data for Cotinine
(X = concentration in $\mu g\ ml^{-1}$, Y = (a) peak height in mm, (b) peak height ratio, (c) peak area in counts x 10^{-6} and (d) peak area ratio)

(a) $\hat{Y} = 5.175\ 3 + 7.8568\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	157.3	162.3123	176.74	or	147.88	194.08	or	130.54
10.0	86.2	83.7438	90.24	or	77.24	106.10	or	61.38
9.0	85.5	75.8869	82.01	or	69.77	97.79	or	53.99
7.0	59.0	60.1732	66.02	or	54.32	81.75	or	38.59
5.0	45.3	44.4595	50.75	or	38.17	66.57	or	22.35
4.0	37.9	36.6027	43.34	or	29.86	59.24	or	13.96
2.0	16.9	20.8890	28.87	or	12.91	45.00	or	-3.22
1.0	9.0	13.0321	21.75	or	4.31	38.02	or	-11.96

(b) $\hat{Y} = 0.0278 + 0.0955\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	1.9220	1.9396	2.01	or	1.87	2.10	or	1.78
10.0	0.9741	0.9837	1.01	or	0.95	1.09	or	0.87
9.0	0.9070	0.8881	0.92	or	0.86	1.00	or	0.78
7.0	0.7405	0.6969	0.73	or	0.67	0.81	or	0.59
5.0	0.5016	0.5057	0.54	or	0.48	0.62	or	0.40
4.0	0.4253	0.4102	0.44	or	0.38	0.52	or	0.30
2.0	0.2012	0.2190	0.26	or	0.18	0.34	or	0.10
1.0	0.0953	0.1234	0.16	or	0.08	0.24	or	0.00

(c) $\hat{Y} = -33109.9195 + 145545.7475\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	2.8784	2.8778	2.96	or	2.79	3.06	or	2.69
10.0	1.4753	1.4223	1.46	or	1.38	1.55	or	1.29
9.0	1.2384	1.2768	1.31	or	1.24	1.40	or	1.15
7.0	0.9661	0.9857	1.02	or	0.95	1.11	or	0.86
5.0	0.6766	0.6946	0.73	or	0.66	0.82	or	0.56
4.0	0.5520	0.5490	0.59	or	0.51	0.68	or	0.42
2.0	0.2560	0.2579	0.30	or	0.21	0.40	or	0.12
1.0	0.1335	0.1124	0.16	or	0.06	0.26	or	0.03

(d) $\hat{Y} = -0.0240 + 0.1104\ X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	2.1931	2.1843	2.21	or	2.15	2.24	or	2.12
10.0	1.0768	1.0801	1.09	or	1.07	1.12	or	1.04
9.0	0.9544	0.9697	0.98	or	0.96	1.01	or	0.93
7.0	0.7490	0.7488	0.76	or	0.74	0.79	or	0.71
5.0	0.5207	0.5280	0.54	or	0.52	0.57	or	0.49
4.0	0.4218	0.4176	0.43	or	0.41	0.46	or	0.38
2.0	0.2001	0.1967	0.21	or	0.19	0.24	or	0.16
1.0	0.0961	0.0863	0.11	or	0.07	0.13	or	0.05

TABLE 3.12: Summary of Calibration Data for Nicotine
(X = concentration in μg ml^{-1}, Y = (a) peak height in mm, (b) peak height ratio, (c) peak area in counts x 10^{-6} and (d) peak area ratio)

(a) $\hat{Y} = 3.9536 + 5.7081 X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	113.1	118.1160	132.34	or	103.9	149.44	or	86.80
10.0	65.0	61.0348	67.45	or	54.63	83.08	or	39.00
9.0	63.6	55.3267	61.36	or	49.30	76.92	or	33.74
7.0	44.5	43.9104	49.68	or	38.14	65.19	or	22.63
5.0	34.6	32.4942	38.69	or	26.29	54.28	or	10.70
4.0	21.2	26.7861	33.44	or	20.14	49.11	or	4.47
2.0	13.6	15.3698	23.24	or	7.50	39.14	or	-8.40
1.0	7.1	9.6617	18.25	or	1.07	34.29	or	-14.97

(b) $\hat{Y} = 0.0228 + 0.0694 X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	1.3829	1.4118	1.52	or	1.30	1.65	or	1.17
10.0	0.7353	0.7173	0.77	or	0.67	0.89	or	0.55
9.0	0.6764	0.6478	0.70	or	0.60	0.81	or	0.49
7.0	0.5582	0.5090	0.55	or	0.47	0.67	or	0.35
5.0	0.3834	0.3701	0.42	or	0.32	0.53	or	0.21
4.0	0.2377	0.3006	0.35	or	0.25	0.47	or	0.13
2.0	0.1623	0.1617	0.22	or	0.10	0.34	or	-0.02
1.0	0.0747	0.0923	0.15	or	0.03	0.28	or	-0.10

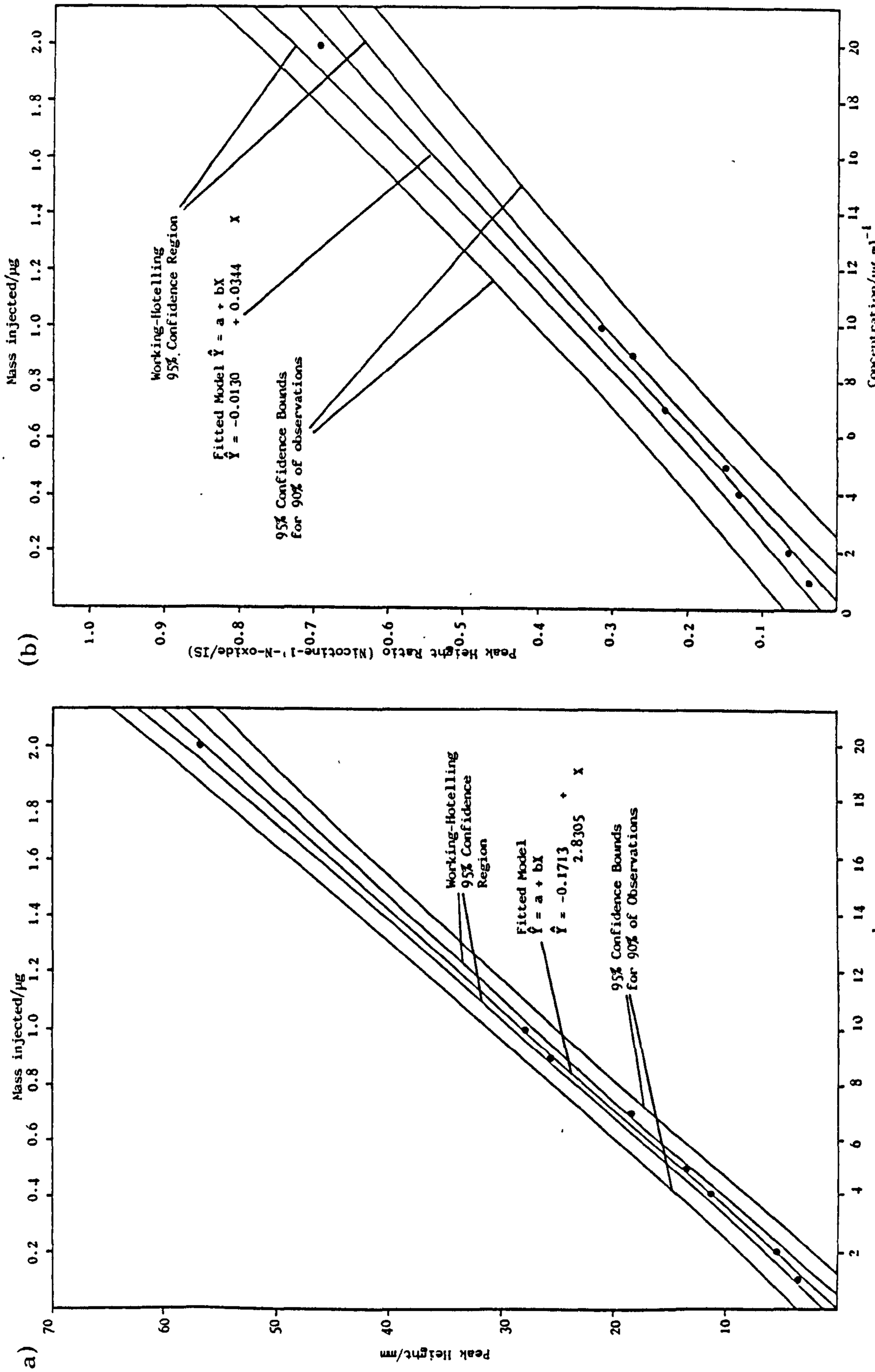
(c) $\hat{Y} = -131950.2162 + 160899.9609 X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	3.1281	3.0860	3.24	or	2.93	3.43	or	2.74
10.0	1.4435	1.4770	1.55	or	1.41	1.72	or	1.24
9.0	1.2973	1.3161	1.38	or	1.25	1.55	or	1.08
7.0	0.9774	0.9943	1.06	or	0.93	1.23	or	0.76
5.0	0.6615	0.6725	0.74	or	0.60	0.91	or	0.43
4.0	0.4269	0.5116	0.58	or	0.44	0.76	or	0.27
2.0	0.2414	0.1898	0.28	or	0.10	0.45	or	-0.07
1.0	0.1001	0.2894	0.12	or	-0.06	0.3	or	-0.24

(d) $\hat{Y} = -0.0990 + 0.1220 X$

X	Y	$\hat{Y}$	Working-Hotelling			Confidence Bounds		
20.0	2.3830	2.3426	2.48	or	2.20	2.64	or	2.04
10.0	1.0540	1.1217	1.18	or	1.06	1.33	or	0.91
9.0	0.9980	0.9997	1.06	or	0.94	1.21	or	0.79
7.0	0.7576	0.7555	0.82	or	0.70	0.96	or	0.56
5.0	0.5087	0.5113	0.57	or	0.45	0.72	or	0.30
4.0	0.3266	0.3892	0.45	or	0.33	0.61	or	0.17
2.0	0.1887	0.1450	0.22	or	0.06	0.37	or	-0.09
1.0	0.0718	0.0230	0.10	or	-0.06	0.26	or	-0.22

Figure 3.3: Calibration curves for Nicotine-1'-N-oxide by (a) Peak height, (b) peak height ratio, (c) peak area and (d) peak area ratio



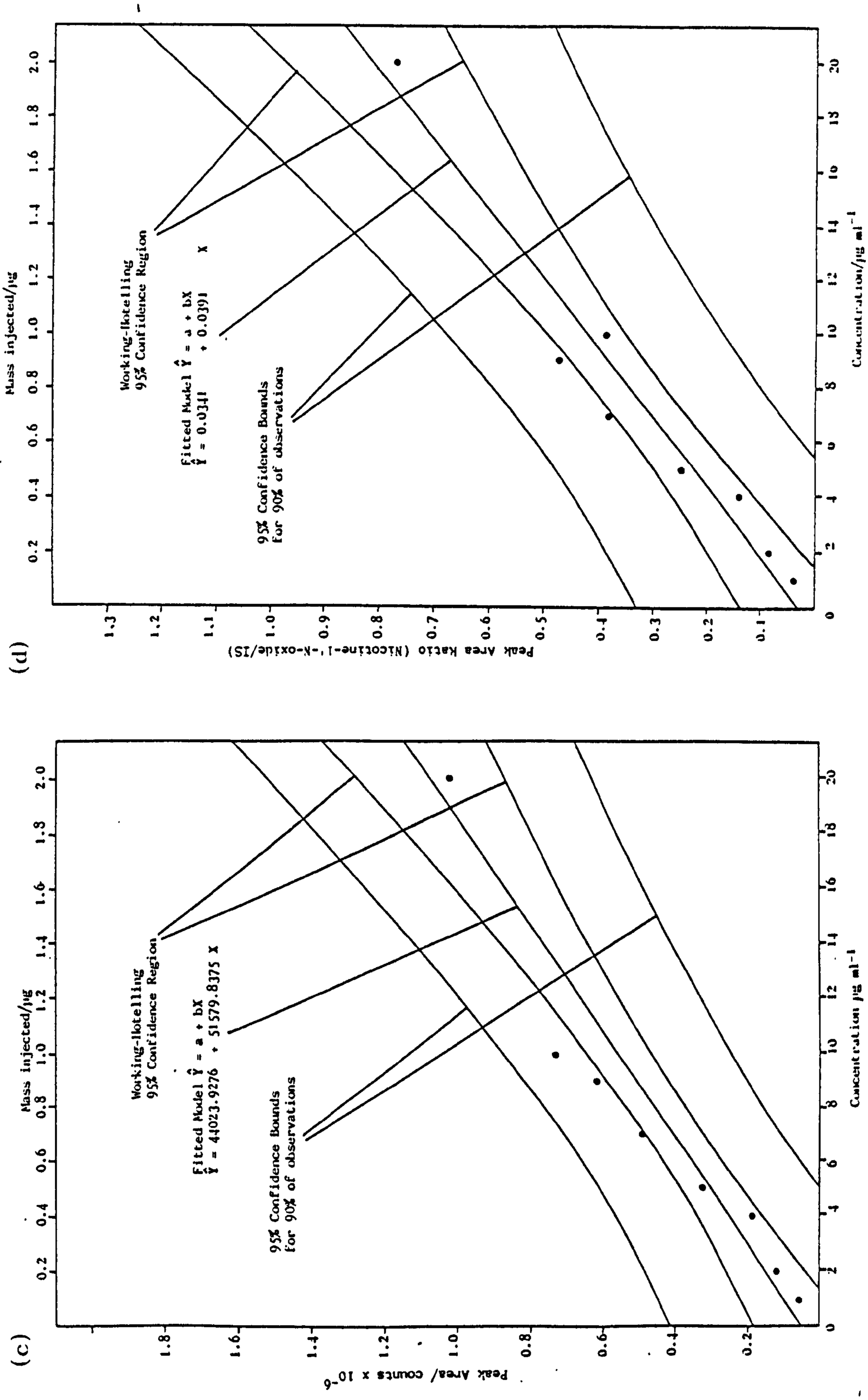


Figure 3.3 (continued)

Figure 3.4: Calibration curves for 3' Hydroxycotinine by (a) Peak height, (b) peak height ratio, (c) peak area and (d) peak area ratio

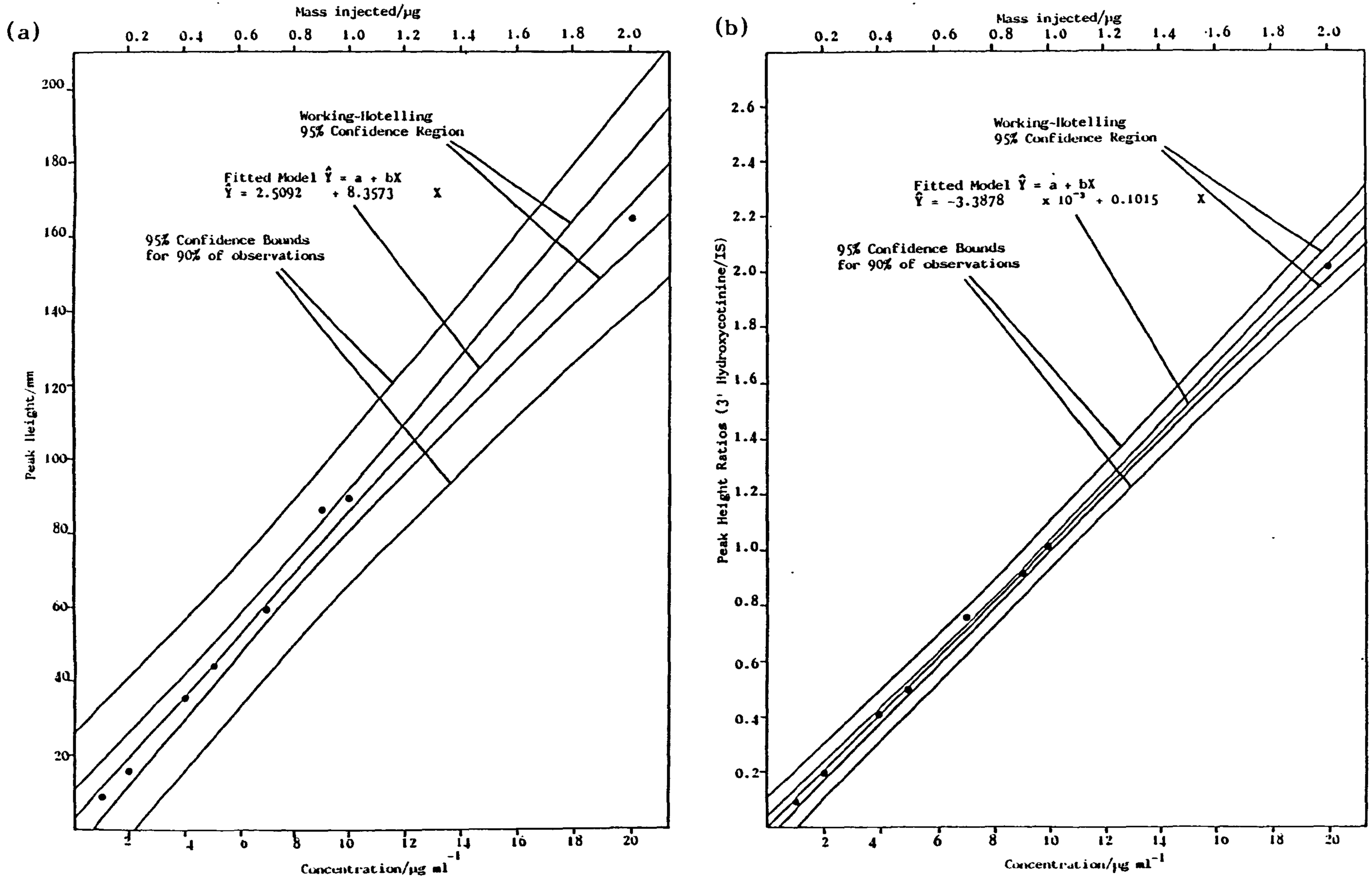


Figure 3.4 (continued)

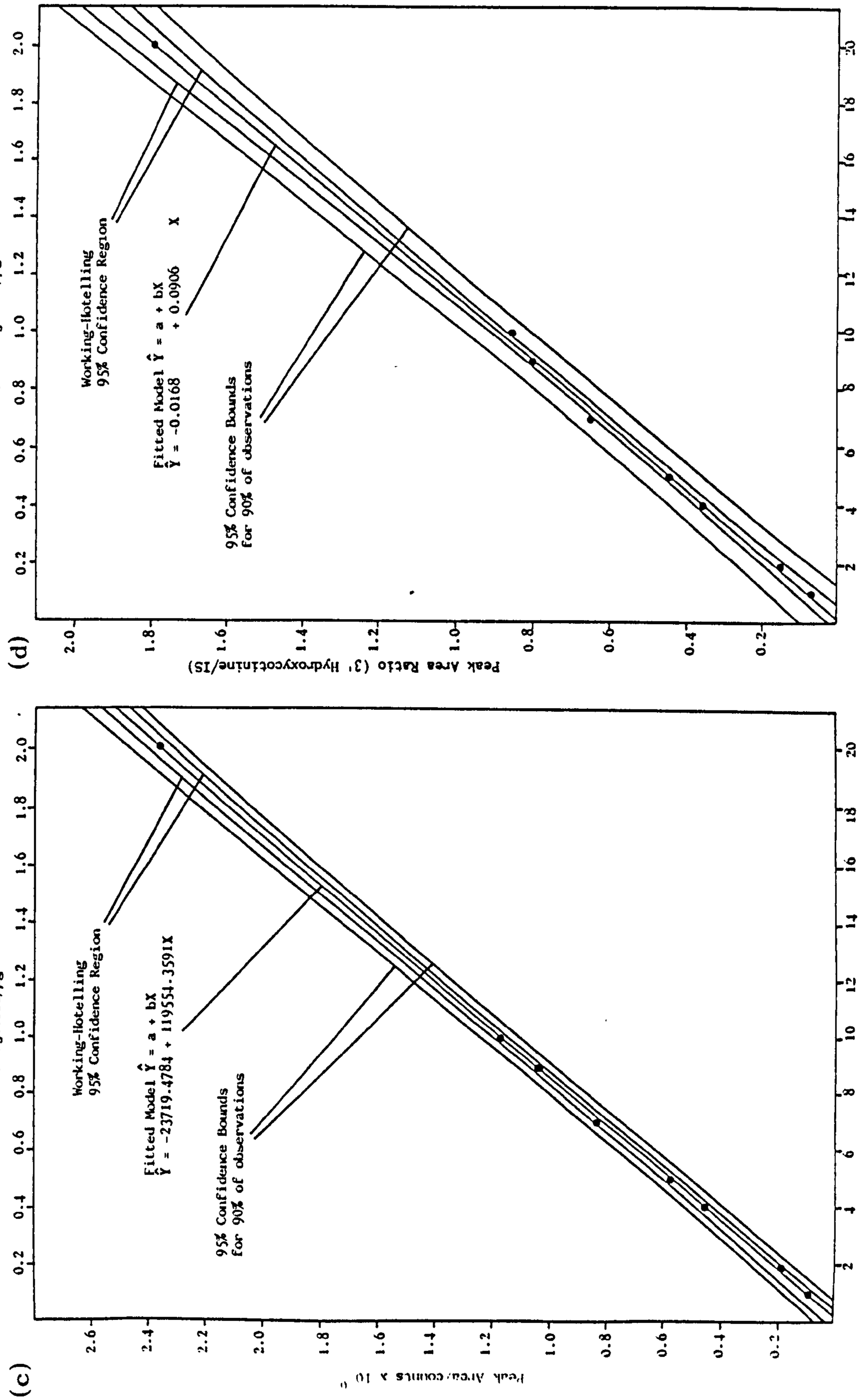


Figure 3.5: Calibration curves for Cotinine by (a) Peak height, (b) peak height ratio, (c) peak area and (d) peak area ratio

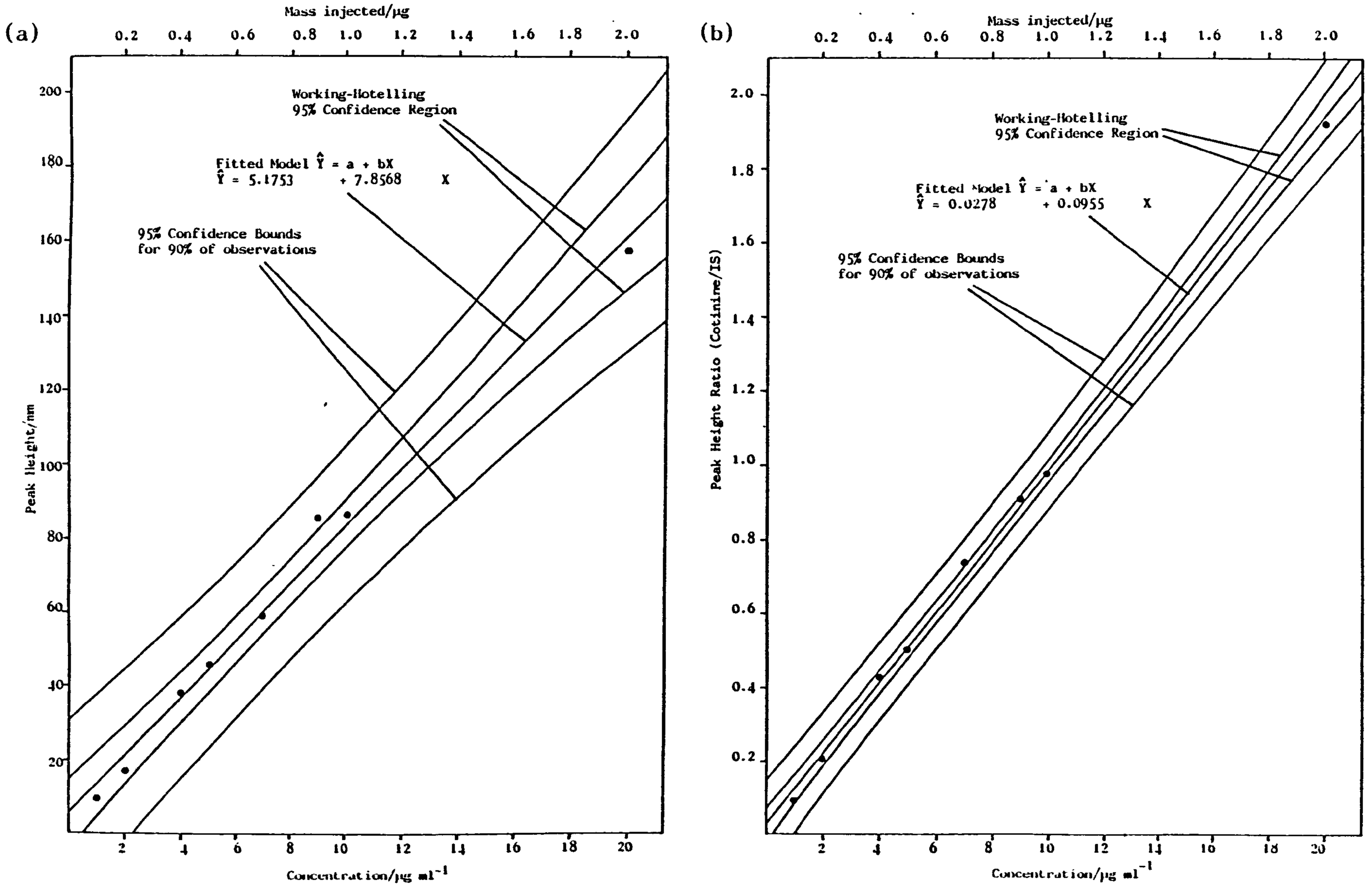
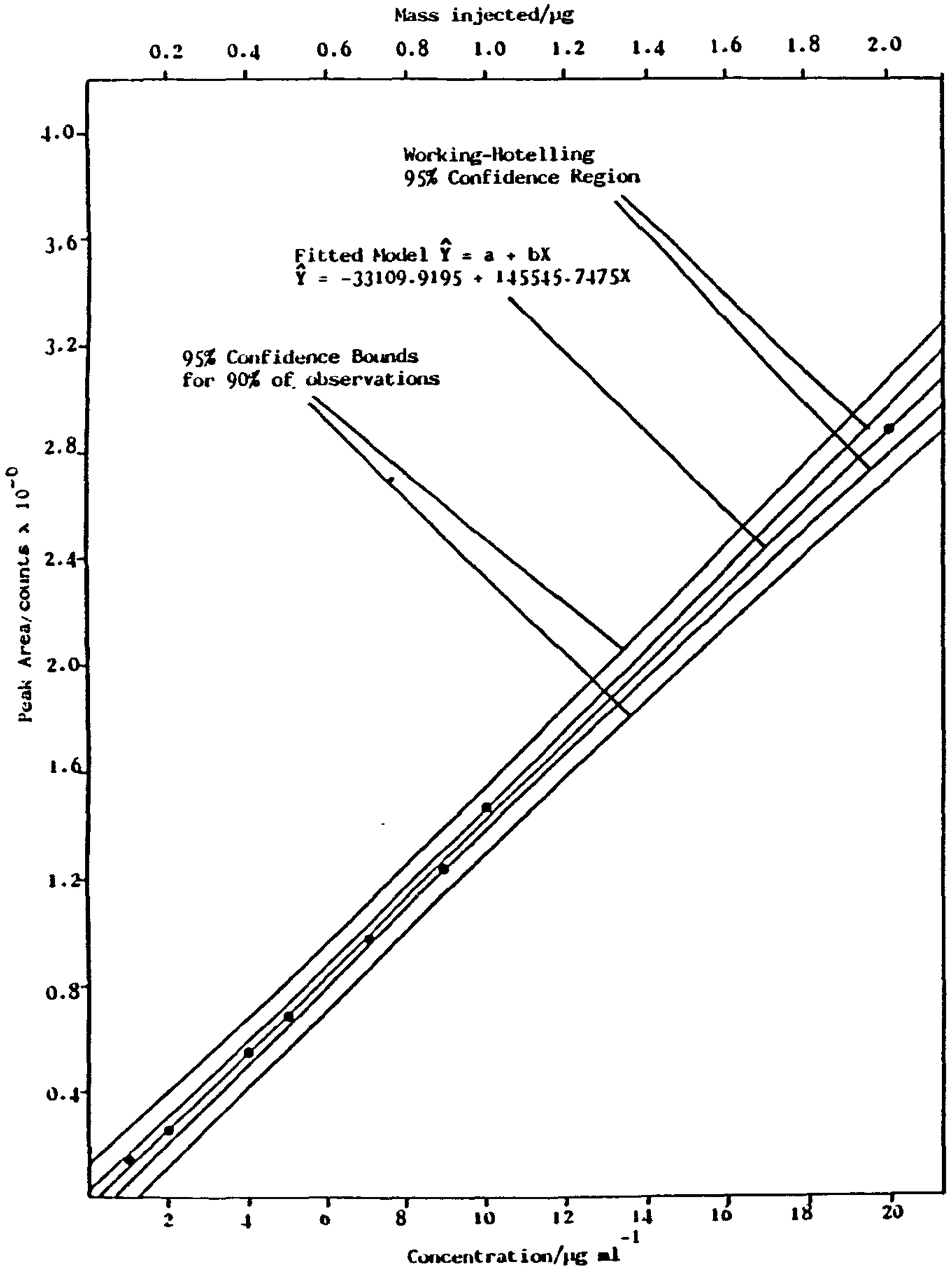


Figure 3.5 (continued)

(c)



(d)

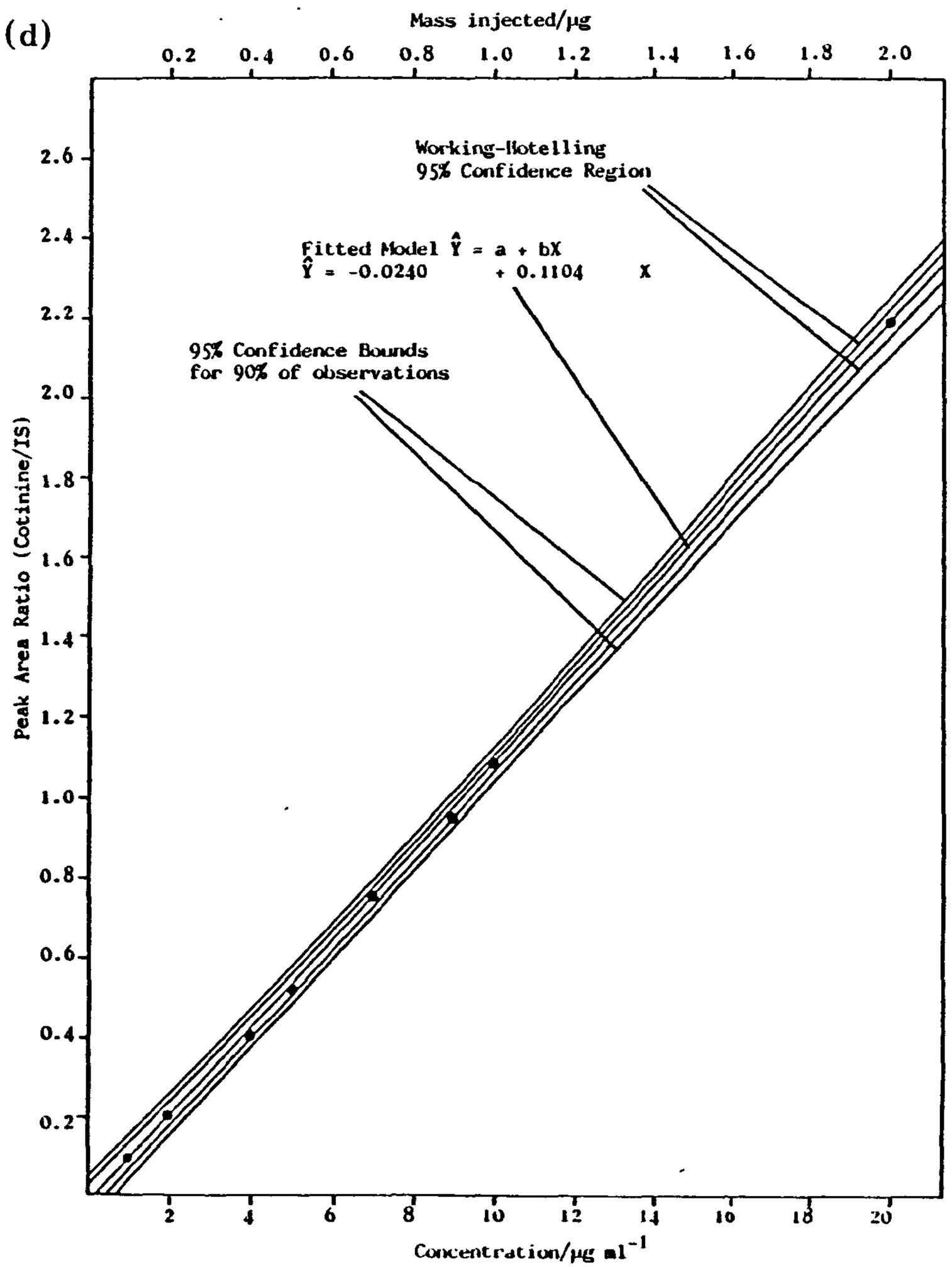
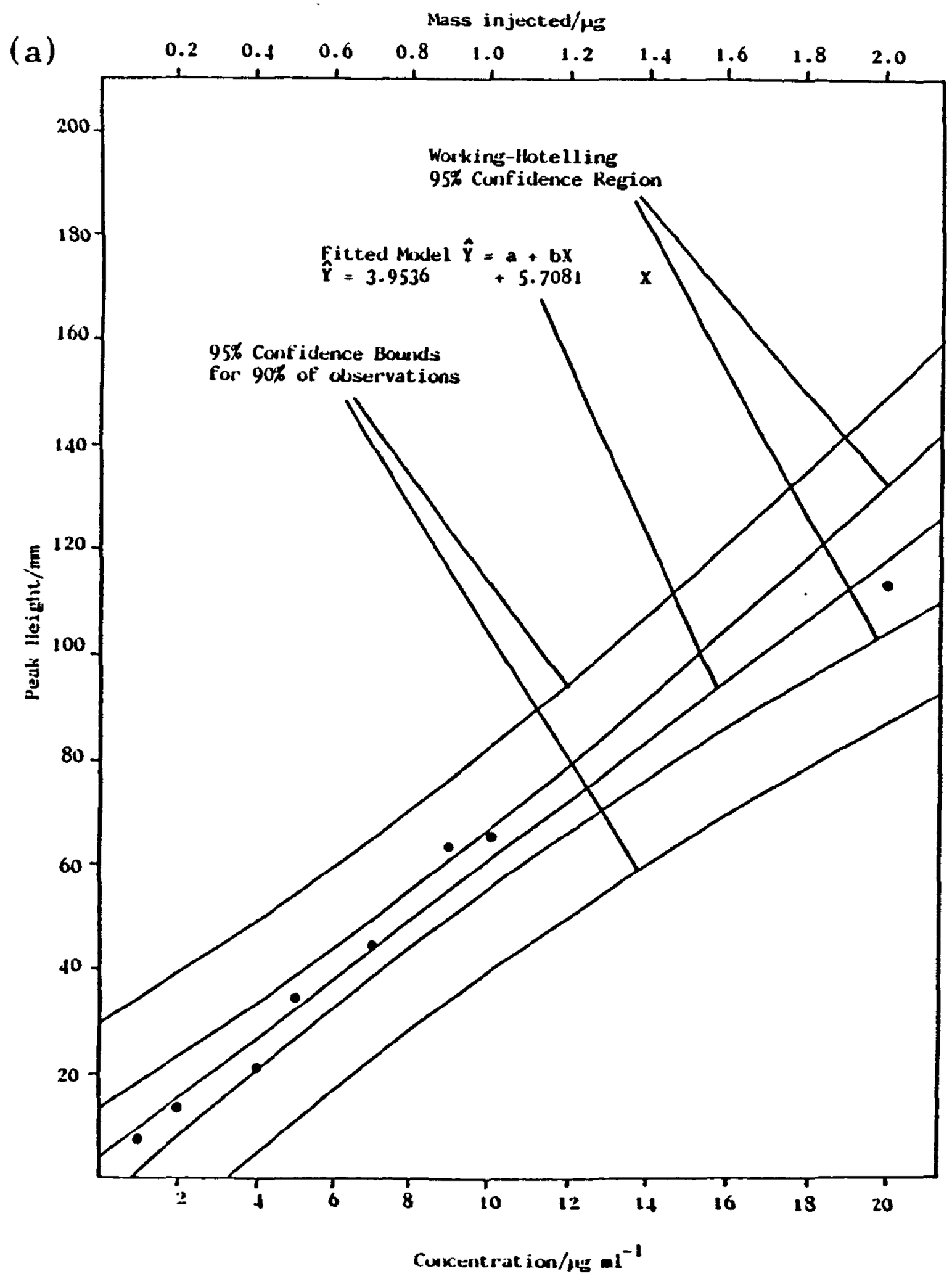
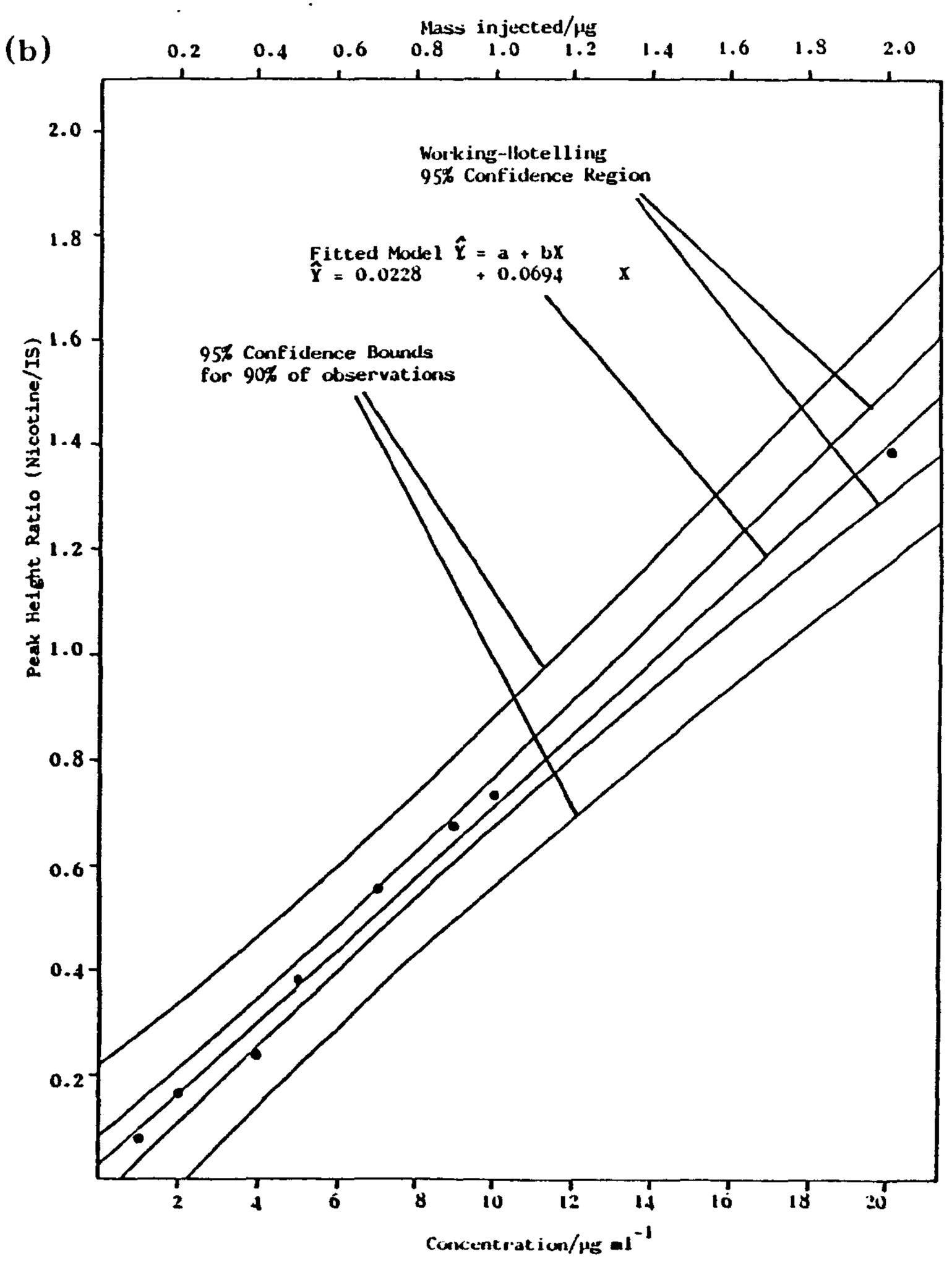


Figure 3.6: Calibration curves for Nicotine by (a) Peak height, (b) peak height ratio, (c) peak area and (d) peak area ratio





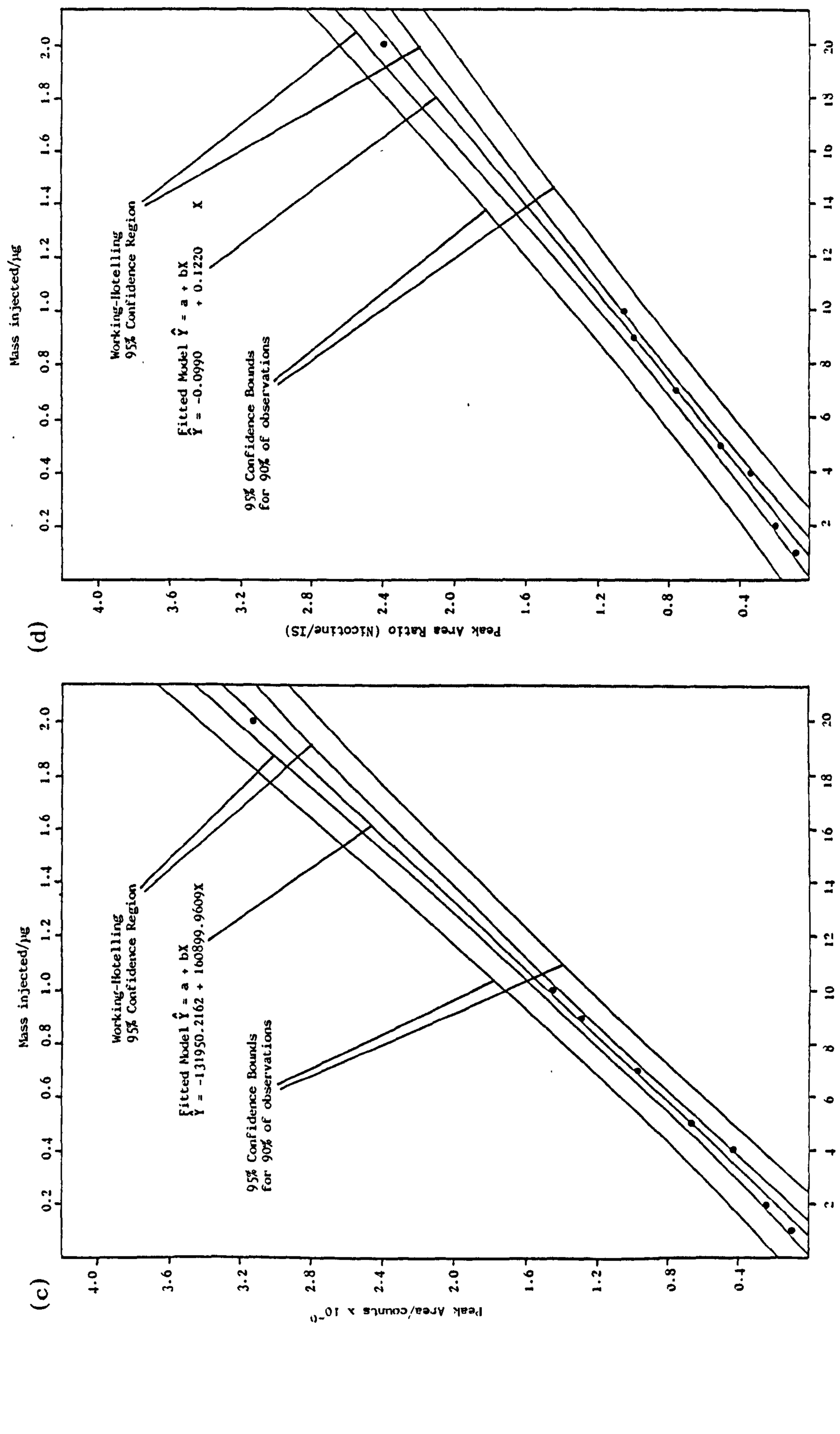


Figure 3.6 (continued)

3.5 Statistical Determination of the Limit of Detection

A commonly used definition of the limit of detection is the analyte concentration giving a signal equal to the blank signal, y_B, plus two standard deviations of the blank, s_B. It is generally agreed that the determination of the limit of detection (LOD) is statistical in nature. However, since the detector response observed on a blank is virtually a horizontal straight line, this precludes measurement of background in units of height or area, the units in which quantitation is performed. The limit of detection must be calculated from other available information, i.e. the calibration data.

Hubaux and Vos [203] have reported a statistical treatment of linear calibration curves which allows calculation of the limit of detection. In 1978, Bailey et al. [204] presented a variation on this approach, illustrated below.

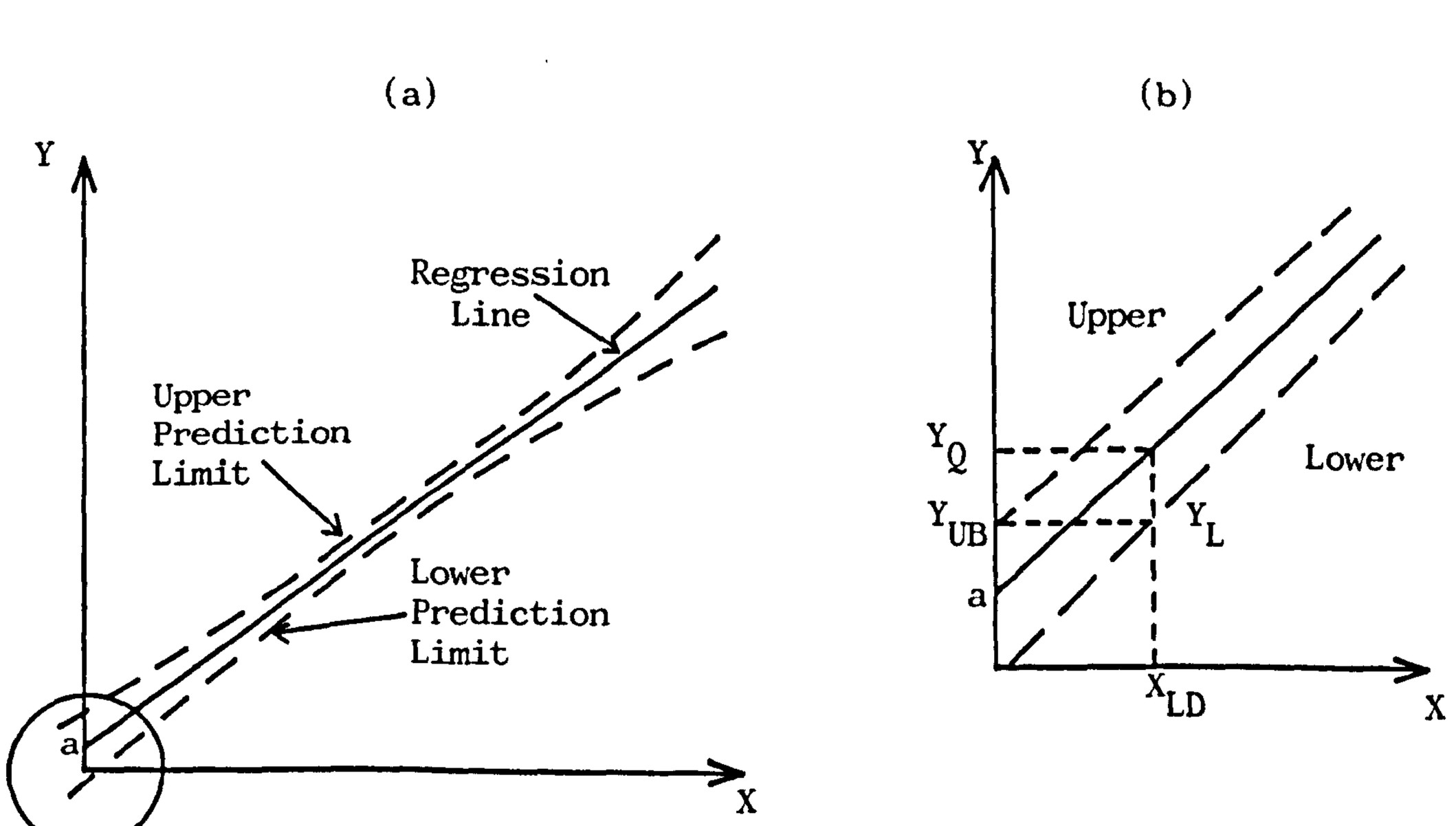


Figure 3.7: (a) Regression line with prediction limits and a, the expected blank value

(b) Enlarged section of (a) showing the parameters a, Y_{UB}, Y_Q, Y_L and X_{LD}.

The regression line, which has been calculated from concentrations and corresponding peak response of the calibration data by the method of least squares, is solved for a concentration of zero to yield a peak area (peak height etc.) which is the expected blank value, denoted by a in figures 3.7. In figure 3.7(b), Y_{UB} is the upper prediction limit on an expected blank value a. Y_L is the lower limit on that predicted individual concentration which exceeds the 99% upper prediction limit on the expected blank, Y_{UB}. X_{LD} is the limit of detection in concentration for a particular substance based on its calibration data, that is, the lowest amount in units of concentration that can be measured above the blank. Y_Q is the area calculated from the regression line which corresponds to X_{LD}. This value is the peak area response above which quantitation may be performed.

The limit of detection is calculated by comparing two prediction limits. The lower prediction limits are compared with the upper prediction limit on the blank until a lower prediction limit is found which is greater than or equal to the upper prediction limit on the blank. A 99% confidence level has been used in this work and Y_{UB}, a 99% upper prediction limit on the blank, is calculated from:

$$Y_{UB} = a + bX + \left[t_{\nu,\alpha}. \, s_{YX} \left(1 + \frac{1}{n} + \frac{(\bar{X})^2}{\Sigma x^2}\right)^{\frac{1}{2}} \right]$$

where t = student's t statistic (Tables)

s_{YX} = standard error of the estimate

$$= \left[\frac{\Sigma y^2 - b\Sigma xy}{n-2} \right]^{\frac{1}{2}}$$

Y_L, the 99% lower prediction limit on the expected area at a given concentration, is calculated from:

$$Y_L = a + bX - \left[t_{\nu,\alpha}. s_{YX} \left(1 + \frac{1}{n} + \frac{(X_p - \bar{X})^2}{\Sigma x^2}\right)^{\frac{1}{2}} \right]$$

Values for X_p are substituted into the above equation. The lowest value of X_p which gives a value for Y_L exceeding or equalling Y_{UB} is the lower limit of detection, X_{LD}, for a single analysis.

A specimen calculation of X_{LD} for nicotine-1'-N-oxide, quantitated by peak height measurements, has been included at the end of this section. The LOD has been calculated for each standard component and by each method of quantitation and is reported in table 3.13. The peak height ratio measurement appears to give the best LOD values over all four standard components. The peak area and peak area ratio measurement parameters for nicotine-1'-N-oxide gave unacceptably high values for LOD. This may again be explained by the uncertainty in the recognition of the start and end-points of this peak in particular. The nicotine-1'-N-oxide peak is the smallest peak and it is not completely resolved from the next eluting peak, 3'hydroxy-cotinine.

The high LOD values calculated give cause for concern. The levels of nicotine and its metabolites in a smoker's urine sample are not expected to exceed 3 $\mu g\ ml^{-1}$. Fortunately sample size is not a problem and also during the extraction of the components of interest from the sample matrix a concentration step may be included, however, it is clear that quantitation of non-smokers' and passive smokers' samples may be extremely difficult.

Statistical Calculation of the Detection Limit for Nicotine-1'-N-oxide Measured by Peak Height

Y_{UB}, the 99% upper prediction limit on the blank, is given by:

$$Y_{UB} = a + bX + \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(\bar{X})^2}{\Sigma x^2}\right)^{\frac{1}{2}} \right]$$

Since $n = 8$, $\nu = n-2 = 6$, and $\alpha = 0.01$,

$t_{6,0.01} = 3.71$ (Tables).

$a = -0.171330723$ $\quad\quad$ $\bar{X} = 7.25$

$b = 2.830528376$ $\quad\quad$ $\Sigma x^2 = 255.5$

$s_{YX} = 0.695446918$

Then at $X = 0$

$$Y_{UB} = -0.171330723 + 2.830528376 \times 0 + \left[3.71 \times 0.695446918 \left(1 + \frac{1}{8} + \frac{(7.25)^2}{255.5}\right)^{\frac{1}{2}} \right]$$

$$\underline{Y_{UB} = 2.805004902}$$

Y_L, the 99% lower prediction limit of the expected response at a given concentration, X_p, is given by:

$$Y_L = a + bX_o - \left[t_{\nu,\alpha} \cdot s_{YX} \left(1 + \frac{1}{n} + \frac{(X_p - \bar{X})^2}{\Sigma x^2}\right)^{\frac{1}{2}} \right]$$

$$Y_L = -0.171330723 + 2.830528376 \times 0 - \left[3.71 \times 0.695446918 \left(1 + 0.125 + \frac{(X-7.25)^2}{255.5}\right)^{\frac{1}{2}} \right]$$

Substituting values for X_p, the equation is solved for Y_L. The lowest value of X_p which yields a value of $Y_L \geq Y_{UB}$ is denoted by X_{LD}.

When $X_p = 2.00$

$Y_L = 2.624903515$ $\quad\quad$ $Y_L < Y_{UB}$

When $X_p = 2.05$

$Y_L = 2.768806904$ $\quad Y_L < Y_{UB}$

When $X_p = 2.06$

$Y_L = 2.797585087$ $\quad Y_L < Y_{UB}$

When $X_p = 2.07$

$Y_L = 2.826362438$ $\quad Y_L > Y_{UB}$

$\therefore \quad \underline{X_{LD} = 2.07\ \mu g\ ml^{-1}}$

Since $V_i = 100\ \mu l$ then $X_{LD} = 0.207\ \mu g$ or 207 ng injected on-column.

TABLE 3.13: Limits of Detection Determined Statistically

Standard	$X_{LD}/\mu g\ ml^{-1}$ (ng)			
	Peak Height	Peak Height Ratio	Peak Area	Peak Area Ratio
Nicotine-1'-N-oxide	2.07 (207)	3.99 (399)	11.60 (1160)	12.62 (1262)
3' Hydroxycotinine	4.64 (464)	1.7 (170)	1.05 (105)	1.88 (188)
Cotinine	5.41 (541)	2.27 (227)	1.75 (175)	0.71 (71)
Nicotine	7.32 (732)	4.57 (457)	2.89 (289)	3.35 (335)

CHAPTER 4

DEVELOPMENT AND OPTIMIZATION OF A CLEAN-UP PROCEDURE FOR URINE SAMPLES AND EXTRACTION OF NICOTINE AND ITS METABOLITES PRIOR TO HPLC ANALYSIS

4.1 Introduction

Separation, detection and quantitation of a standard mixture is a relatively straightforward procedure when compared to the analysis of the same mixture of components in a sample, e.g. urine, blood or tissue. Analysis of the sample, which also contains numerous other substances, can prove extremely difficult. Due to the complexity of most samples, direct analysis is not possible and the sample must first undergo additional preseparations to reduce the number of interfering substances. If sample impurities are not removed they can remain on the column, causing blockage or a reduction in column efficiency resulting in a deterioration in the resolving power of the column. Sample impurities can also saturate or contaminate the detector. The less specific the detector the more critical it is to remove interfering materials.

All separation techniques can be considered for the sample clean-up: filtration; centrifugation; precipitation; evaporation; extraction of solids and liquids by liquids; extraction of liquids by adsorption on solids. In most cases, combinations of different extraction mechanisms are required.

The clean-up procedure should separate the analyte(s) from interfering matrix elements and leave them in appropriate concentrations for detection and quantitation.

During all clean-up procedures analyte losses can occur. In order to monitor and correct for these losses an IS may be added to the sample before the preseparation procedures are carried out. The IS must be similar to the component(s) of interest as it will be taken through the isolation steps.

Many separation techniques are available, however only those techniques relevant to the clean-up of urine samples will be considered further.

4.2 Filtration/Centrifugation

Some samples require filtration or centrifugation as part of the clean-up, clear, particle-free solutions being a prerequisite for HPLC analysis. Although these two techniques are similar, centrifugation may be preferred as the sample does not come into contact with the filter material which could adsorb the analyte.

4.3 Deproteinization

Biological samples, such as urine, with a high protein content, make precipitation of the proteins necessary in order to prevent the formation of emulsions during extraction with organic solvents. Deproteinization may cause problems due to the possibility of irreversible binding of the analyte to the proteins, in particular where the analyte is lipophilic. An IS may not allow satisfactory adjustment for these losses as this would require the distribution ratio and protein binding of the analyte and the IS to be similar. Extraction by means of solid phases avoids the formation of emulsions and may be used as an alternative so avoiding the need for the more problematic protein-precipitation.

4.4 Liquid-Liquid Extractions

Liquid-liquid extraction is probably the most widely used technique for cleaning up samples prior to analysis. It is a relatively simple and rapid procedure and usually results in high purification.

The solvents chosen should form two immiscible phases after equilibration and the components of interest must strongly favour distribution into one phase, while the impurities favour distribution into the other.

Although the solvents should be immiscible, water may dissolve in the organic phase and hence polar compounds may be transferred to the organic phase in the dissolved water, thus the extract should be washed several times with small amounts of pure aqueous phase.

The extraction yield is related to the distribution ratio of the component of interest between the two phases. In many cases the distribution ratio is pH dependent, and therefore the most favourable pH range for extraction must be chosen. Components having ionizable groups can be most efficiently extracted into the organic phase from the aqueous phase when ionization is suppressed. The uncharged species is predominant in the pH region below the pK_a for acids, above the pK_a for bases and at the isoelectric pH for amphoteric compounds. Components which remain unionized over a wide pH range can be purified by extracting the contaminating compounds into the aqueous phase by the same procedure.

When compounds are hydrophilic and charged, extraction into the organic phase will be inefficient as the distribution ratio will be small. The extraction efficiency can often be improved

if the compound is extracted as a neutral complex that is more soluble in the organic phase. This technique has been widely used in the extraction of very polar analytes from body fluids.

4.5 Liquid-Solid Extractions

Whenever distribution problems exist, solid adsorption methods also provide reasonably pure extracts. A wide variety of solid adsorbents is now available including silica, alumina and reversed phase materials. The extraction procedure involved an adsorbent of appropriate activity being packed into a column and the sample solution, applied to the inlet, being allowed to flow into the adsorbent bed under gravity, positive pressure or vacuum at a controlled flow rate. Pre-packed, disposable cartridges are now readily available e.g. Sep Pak or Bond Elut.

The adsorbent and solvent may be chosen so that the components of interest are unretained by the column/cartridge and the interfering components are adsorbed. This procedure may be preferred when the sample component of interest is present in a high concentration. When components of widely differing polarities need to be isolated or they are present in low levels, then an adsorbent/solvent system which allows the matrix interferences to pass through unretained but adsorbs the components of interest may be the most effective. A series of solvents or solvent mixtures of increasing elution strength is then used to desorb selectively the desired components.

The formation of neutral complexes can again be used in the

extraction of very polar components.

Generally, the recovery of the components of interest from liquid-solid extraction procedures is superior to that from liquid-liquid extraction, however the number and quantities of co-extracted constituents is also greater. Therefore, more isolation steps may be necessary after liquid-solid extraction in order to obtain a clean extract.

Although the optimization of liquid-solid extractions might be complicated they are advantageous for routine analysis, being relatively simple to perform and requiring only small sample volumes; they are also amenable to automation.

4.6 Concentration Steps

Samples can often be concentrated by extraction into a smaller volume of solvent, however in many cases the final volume after extraction may exceed the original sample volume due, for example, to several washings of the organic phase with an aqueous solution in order to improve the extraction yield, requiring the final extract to be concentrated prior to HPLC analysis.

Although evaporation is a simple solution it can result in loss of the components of interest through degradation, oxidation or volatilization. Evaporation using nitrogen streaming through needles over the surface of the solvent lowers the evaporation temperature and so may prevent loss of the components of interest.

4.7 Calibration and Quantitation

Once the extraction procedure has been finalized, calibration curves over the expected concentration range should be constructed for samples with known concentrations of components added and processed through the whole extraction procedure.

4.8 Development of a Clean-up Procedure

4.8.1 Preliminary Experiments

Initially, a clean-up procedure (see figure 4.1, extraction procedure 1), previously used prior to GC analysis of nicotine and cotinine,[182] was applied to the samples to be analyzed by HPLC. A standard solution was extracted giving the following recovery values: nicotine, 86%; cotinine, 90%; anabasine, 80%; and nicotine-1'-N-oxide only 48%; see figure 4.2. When the extraction procedure was applied to blank (non-smokers') urine samples, it was obvious that the clean-up was not sufficiently good when the subsequent analysis was to be carried out by HPLC with UV detection. A chromatogram of the extracted blank urine sample is presented in figure 4.3. The GC analysis referred to involved the use of a capillary column and a nitrogen sensitive alkali tip detector which provide efficient chromatography and an enhanced response to compounds containing nitrogen, so reducing the importance of the extraction and clean-up procedures required.

Figure 4.2: Extraction Procedure 1 (Figure 4.1) applied to a standard mixture (10 μg ml^{-1}) in H_2O.
Parameters: Column: Resolve C_{18} 5 μ Radial PAK Cartridge (10 cm x 8 mm ID) in the RCM-100. Detection: UV at 260 nm. Flow Rate: 1.7 ml min^{-1}. Eluent: Liquid A: 0.2% H_3PO_4, pH adjusted to 7.25 with Et_3N; Liquid B: MeOH. Gradient programme # 5, 3 min initial hold at 27%B, 27% → 58%B over 14 mins. Sample Size: 100 μl.

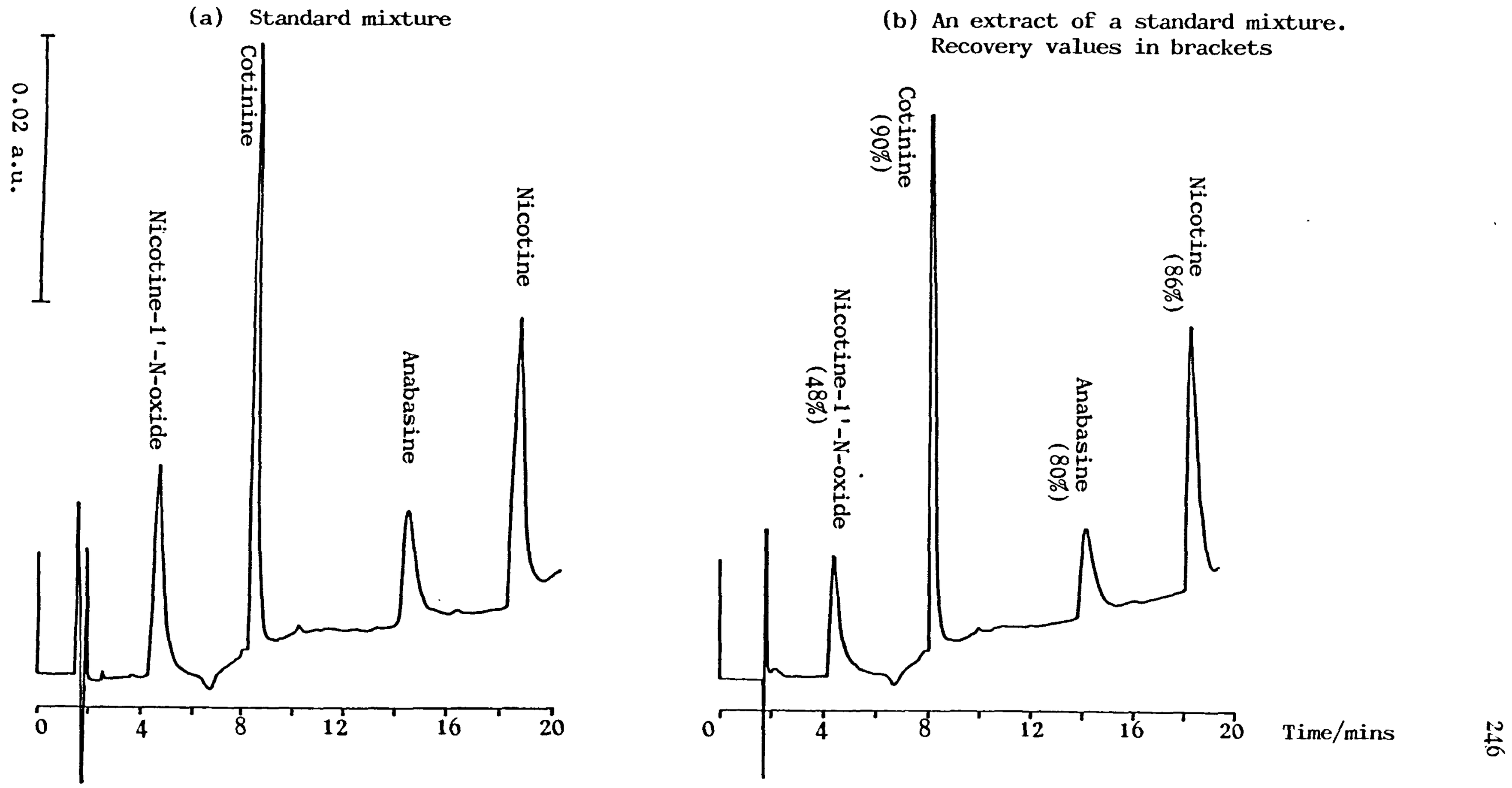


Figure 4.3: Extraction Procedure 1 (figure 4.1) applied to a urine sample (female, non-smoker)
Parameters: see figure 4.2

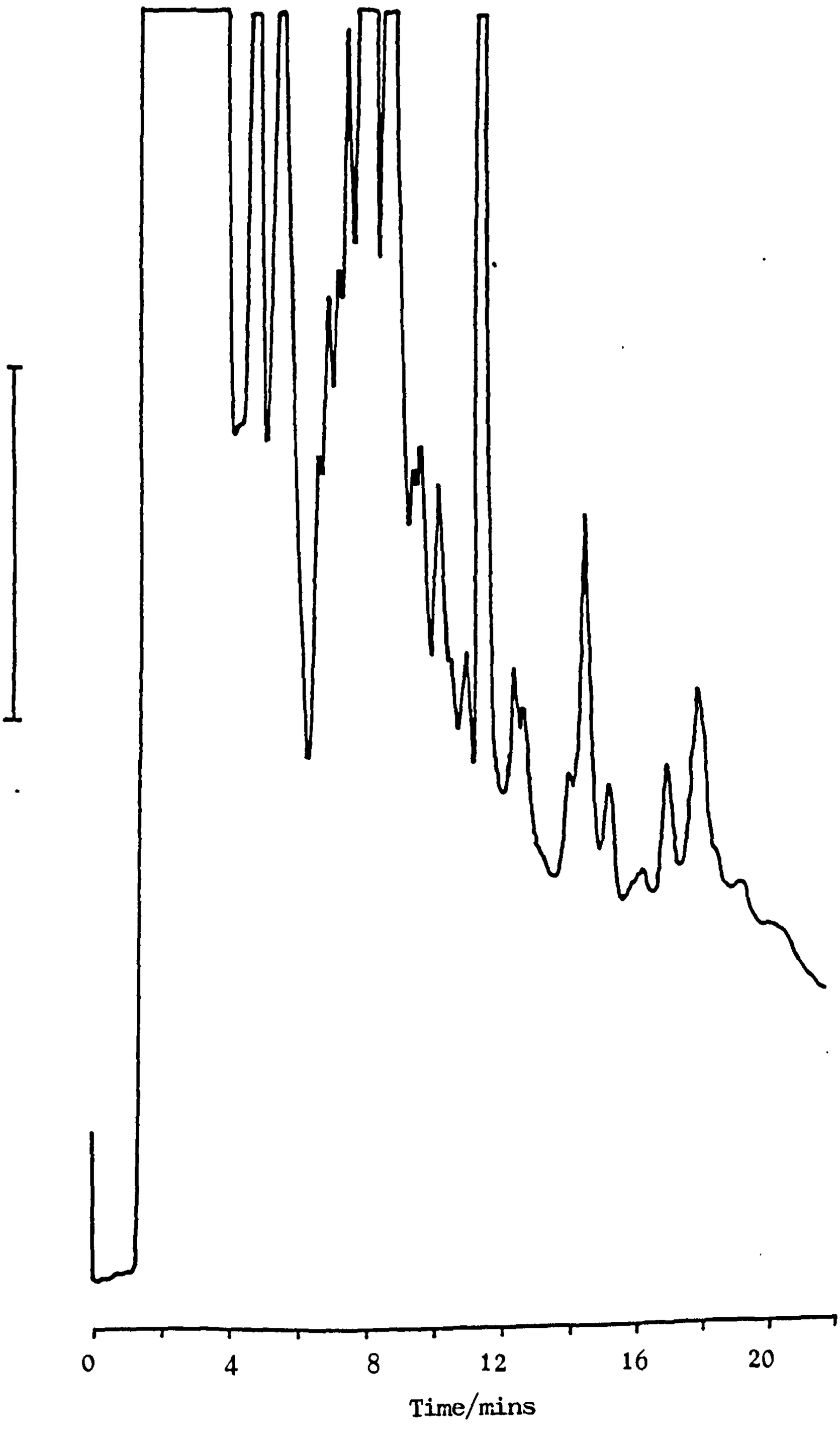


Figure 4.1: Extraction Procedure 1

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 7.0 using HCl or NaOH

↓

*Pass sample (10 ml) through a Sep Pak and discard effluent (ODS)

↓

Wash Sep Pak with H_2O (2 ml) and discard

↓

Elute components of interest from Sep Pak with 100% methanol (5 ml)

↓

Make extract volume 10 ml by adding 0.2% H_3PO_4, pH 7.25

↓

Inject

* All extractions carried out in duplicate

An extraction procedure carried out at pH 7.0 using dichloromethane was tried as an alternative to using a Sep Pak, see figure 4.4, extraction procedure 2. An experiment on a standard mixture revealed that nicotine-1'-N-oxide was not extracted using dichloromethane and the recovery values for nicotine and cotinine were

49% and 68% respectively. Nicotine-1'-N-oxide being hydrophilic remained in the aqueous layer, as illustrated in figure 4.5.

Figure 4.4: Extraction Procedure 2

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 7.0 using HCl or NaOH

↓

Extract components of interest from sample (10 ml) using CH_2Cl_2 (2 x 10 ml)

↓

Evaporate to dryness using N_2 stream

↓

Reconstitute by the addition of MeOH (5 ml) and 0.2% H_3PO_4, pH 7.25 (5 ml), total volume, 10 ml

↓

Inject

Another extraction procedure using both dichloromethane and a Sep Pak was investigated, see figure 4.6, extraction procedure 3. At pH 3.0, the components of interest were expected to remain in the water layer, while some of the impurities favoured distribution into the organic solvent, dichloromethane. Readjusting the pH to 7.0 allowed further clean-up when the sample was passed through the Sep Pak. The components are retained by the Sep Pak until they are removed by a 100% MeOH wash. Although good extraction yields for cotinine (76%) and nicotine (85%) were obtained, the

Figure 4.5: Extraction Procedure 2 (figure 4.4) applied to a standard mixture (10 μg ml^{-1}) in H_2O. **Parameters:** see figure 4.2 except for Gradient programme #5, 3 min initial hold at 25%B, 25%B - 58%B over 15 mins

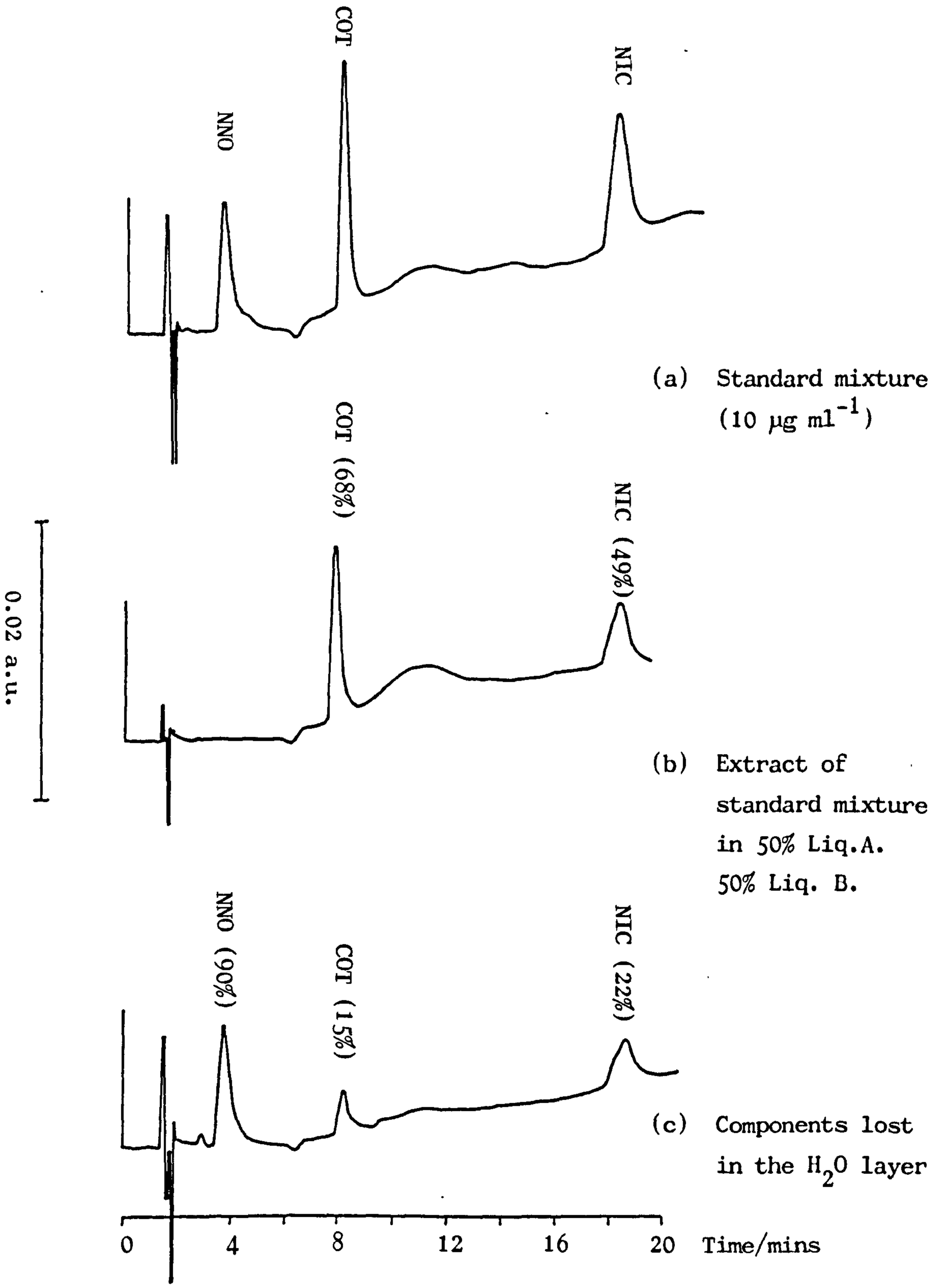


Figure 4.6: Extraction Procedure 3

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 3.0 using HCl

↓

Extract sample (10 ml) with CH_2Cl_2 (2 x 10 ml)
discard CH_2Cl_2 fraction

↓

Collect aqueous fraction (10 ml)

↓

Adjust pH to 7.0 using NaOH

↓

Pass aqueous fraction (10 ml) through a
Sep Pak and discard effluent

↓

Wash Sep Pak with H_2O (2 ml) and discard

↓

Elute components of interest
from the Sep Pak with MeOH (5 ml)

↓

Make extract volume 10 ml by adding
0.2% H_3PO_4, pH 7.25

↓

Inject

recovery value for nicotine-1'-N-oxide (41%) was not satisfactory, as shown in figure 4.7. It was hoped that when the extraction procedure was applied to urine samples a cleaner extract would result, however the use of dichloromethane in the clean-up of urine samples caused the formation of emulsions, making deproteinization a necessary consideration.

Extractions by means of a solid phase, such as the use of a Sep Pak, avoids the necessity for protein precipitation and the associated risk of loss of analyte(s) due to protein binding. However, the extraction of a standard mixture at pH 3.0 using a Sep Pak, followed by a further isolation step at pH 7.0, again using a Sep Pak, resulted in poor extraction yields for all the components of interest: nicotine-1'-N-oxide (53%), cotinine (50%) and nicotine (58%), see figure 4.8, extraction procedure 4 and figure 4.9.

Inefficient extractions of less than 50% of a component can lead to poor reproducibility in its recovery as it is perhaps more susceptible to fluctuations in e.g. temperature and sample composition. In general, the more efficient an extraction procedure, the more reproducible it should be.

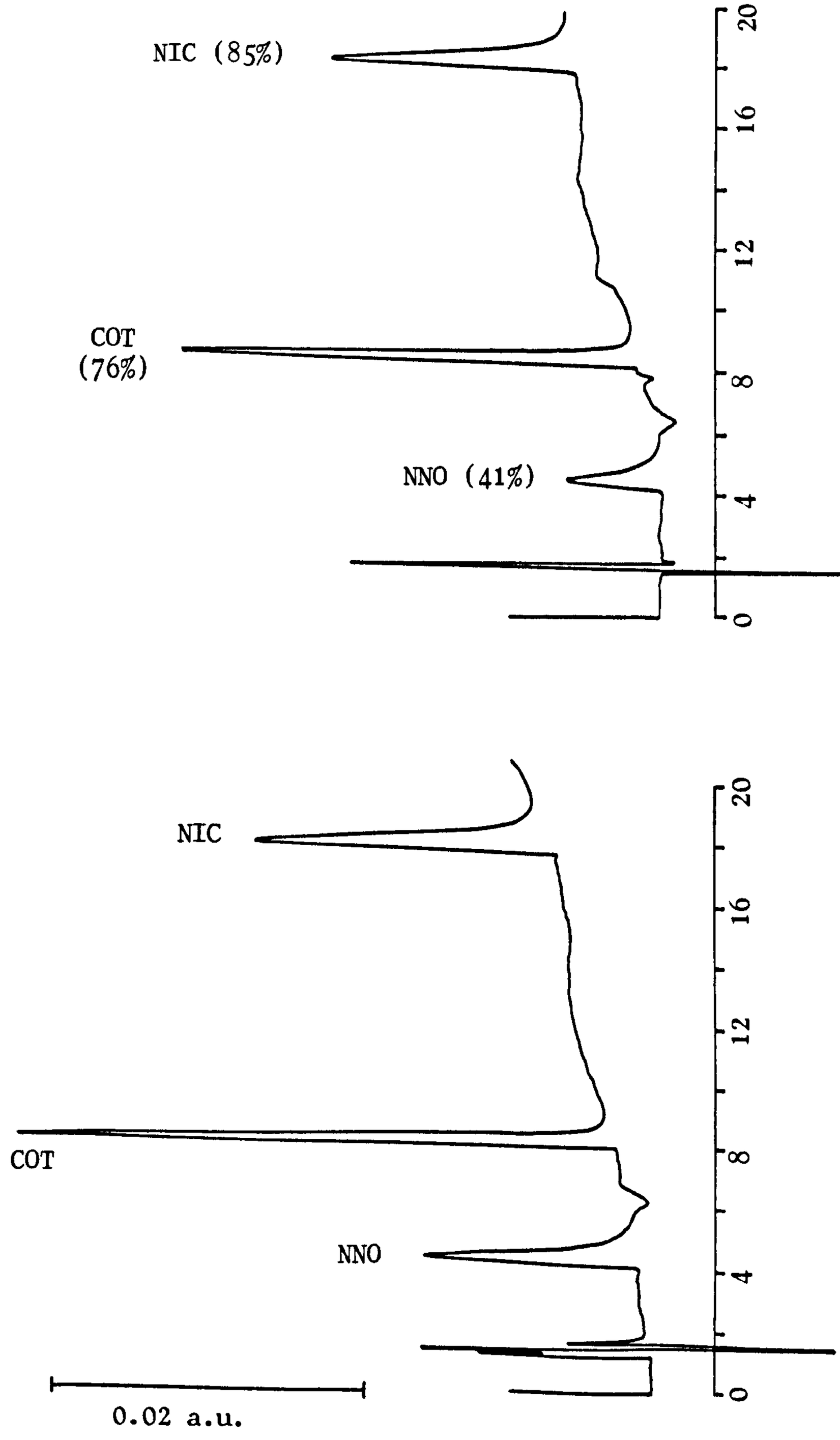
Taking the above results into account, the clean-up of urine samples was not investigated by extraction procedures 2, 3 or 4.

The isolation and analysis of N-oxide metabolites of tertiary amines have been reported by Thompson et al.[169] Nicotine-1'-N-oxide was isolated as a dodecylsulphate ion pair with C_{18} extraction cartridges. The nicotine-1'-N-oxide ion pairs were employed in extractions because the resulting lipophilic species permitted

Figure 4.7: Extraction Procedure 3 (figure 4.6) applied to a standard mixture (10 μg ml^{-1}) in H_2O

Parameters: see figure 4.5

(a) Standard mixture



(b) Extract of a standard mixture

Figure 4.8: Extraction Procedure 4

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 3.0 using HCl

↓

Pass sample (10 ml) through a Sep Pak
and collect effluent

↓

Adjust pH to 7.0 using NaOH

↓

Pass sample (10 ml) through another Sep Pak
and discard effluent

↓

Wash Sep Pak with H_2O (2 ml) and discard

↓

Elute components of interest from the Sep Pak
with 100% methanol (5 ml)

↓

Make extract volume 10 ml by adding
0.2% H_3PO_4, pH 7.25

↓

Inject

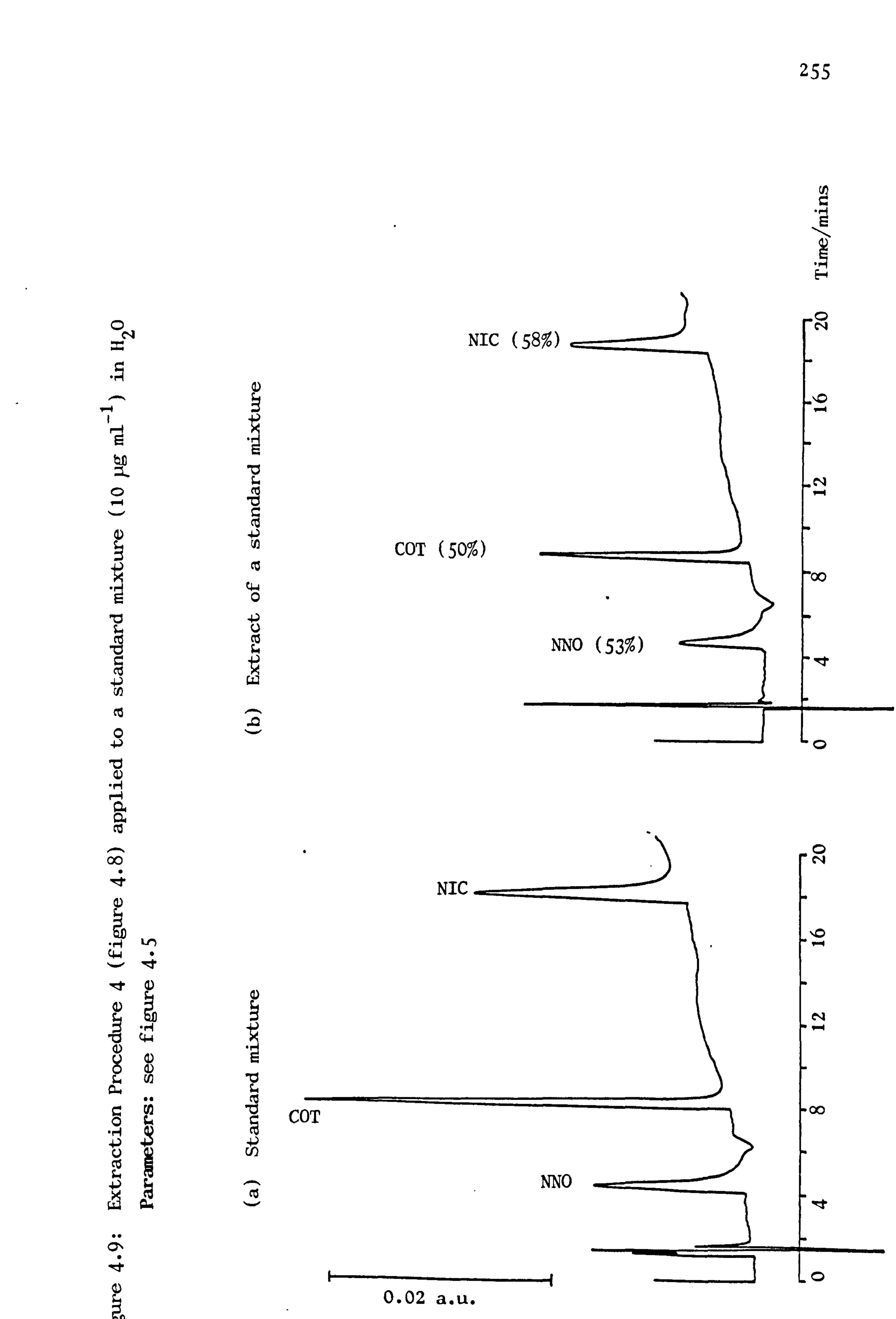


Figure 4.9: Extraction Procedure 4 (figure 4.8) applied to a standard mixture (10 μg ml^{-1}) in H_2O

Parameters: see figure 4.5

(b) Extract of a standard mixture

(a) Standard mixture

washing the Sep Pak cartridges with methanol-water mixtures in order to remove unwanted polar substances before nicotine-1'-N-oxide was finally eluted using 100% methanol. Thompson et al. reported washing the Sep Pak with methanol-water mixtures containing up to 50% methanol, in this study it was found that nicotine, cotinine and nicotine-1'-N-oxide suffered substantial losses if a 50% methanol: 50% water wash was used. A 40% methanol:60% water wash caused the loss of 22% cotinine. A 35% methanol:65% water wash was found to be satisfactory for washing the Sep Pak without causing the loss of any of the components of interest, see figure 4.10, extraction procedure 5 and figure 4.11.

The optimized extraction procedure, 6, is reported in figure 4.12. This scheme proved very successful in extracting the components from a standard mixture in water, as shown in figure 4.13, and resulted in a dramatic improvement in the clean-up of blank urine samples, as illustrated in figures 4.14 and 4.15. However, urine samples, spiked before extraction (≡ 10 $\mu g\ ml^{-1}$ nicotine, cotinine and nicotine-1'-N-oxide) did not give the expected result. Cotinine was not recovered, as shown in figure 4.16. Further investigation of each isolation step in extraction procedure 6, figure 4.12, revealed that cotinine was lost when the spiked urine sample was passed through the Sep Pak initially at pH 2.0 and was not retained by the Sep Pak as expected, see figure 4.17. The reasons why cotinine did not remain on the Sep Pak are not clear. Extraction procedure 6 gave a successful outcome when a standard mixture in H_2O, including cotinine, was examined. However, in the extraction of cotinine from a spiked urine sample at pH 2, cotinine obviously did not

Figure 4.10: Extraction Procedure 5

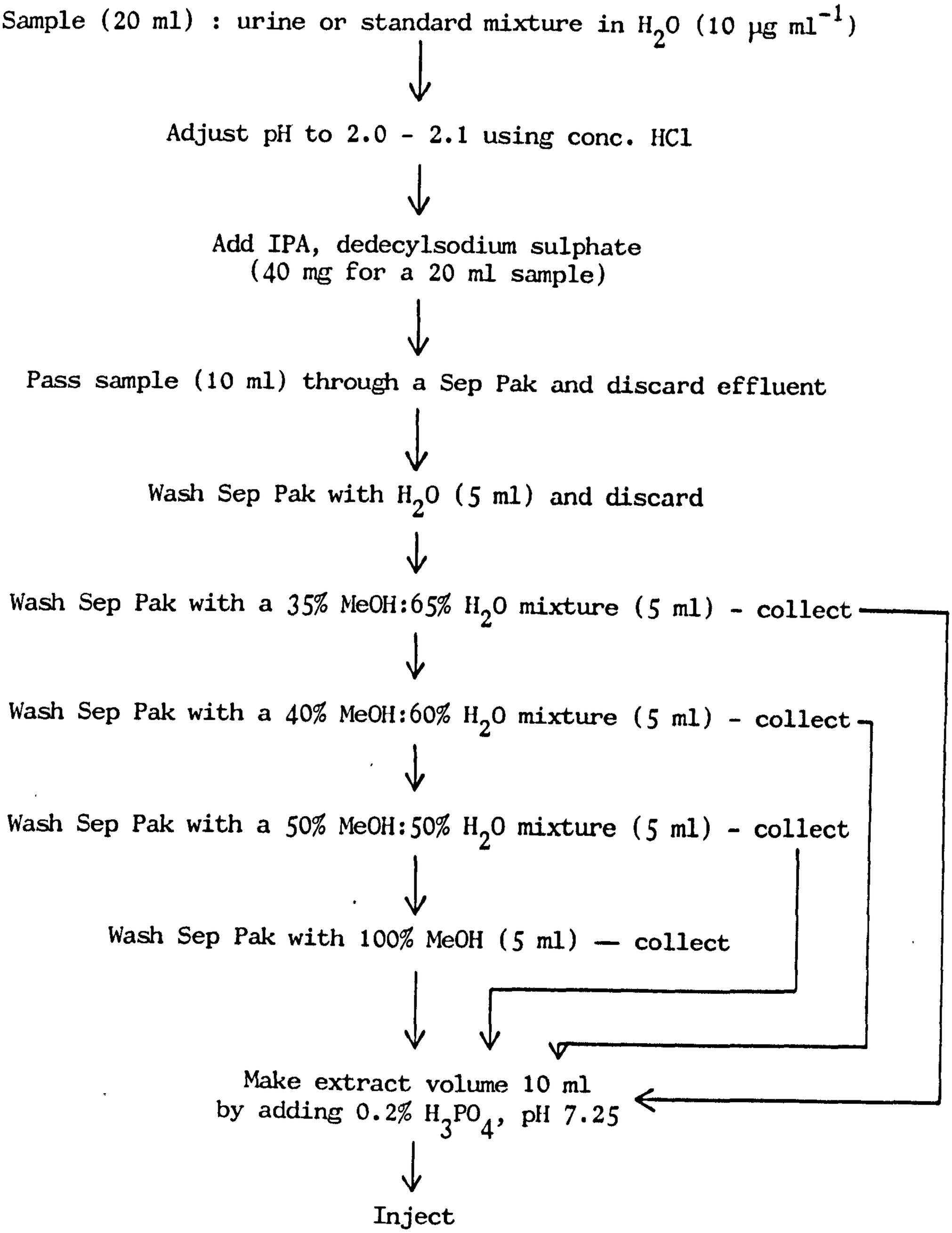
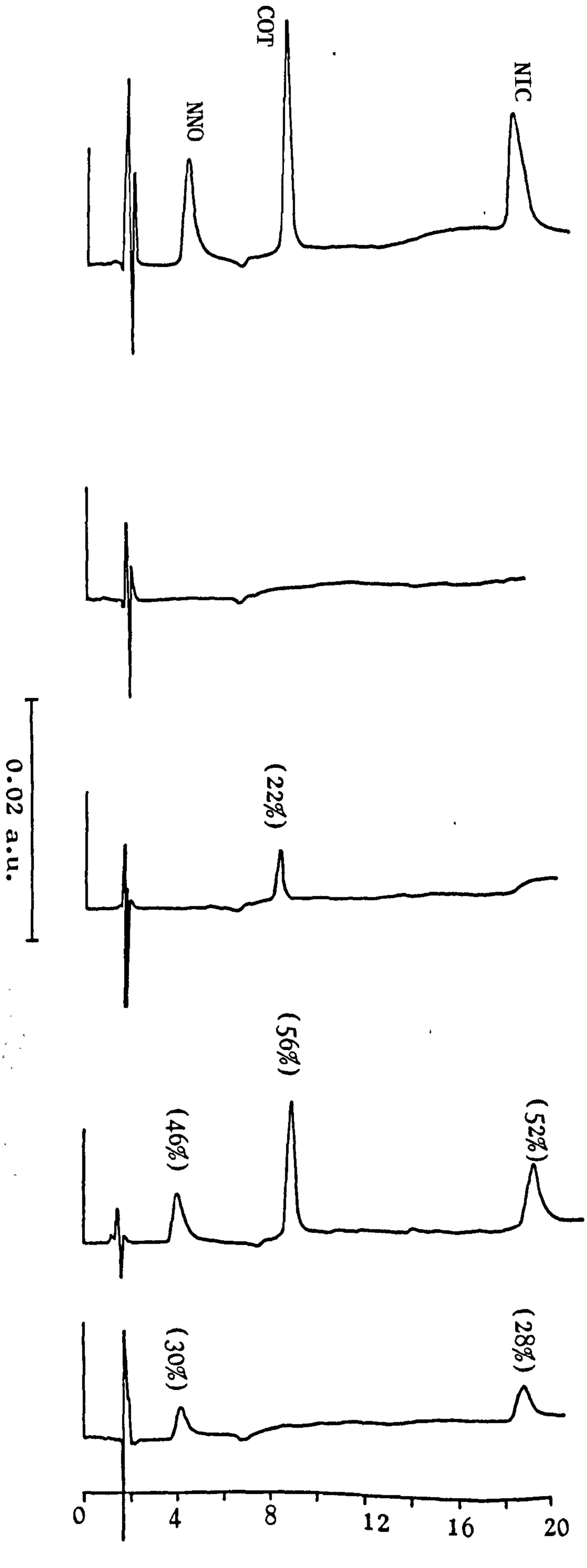


Figure 4.11: Extraction Procedure 5 (figure 4.10) applied to a standard mixture (10 μg ml^{-1}) in H_2O
Parameters: see figure 4.5



(a) Standard mixture

(b) Effluent from a 35% MeOH:65% H_2O wash

(c) Effluent from a 40% MeOH:60% H_2O wash

(d) Effluent from a 50% MeOH:50% H_2O wash

(e) Extract of a standard mixture

Figure 4.12: Extraction Procedure 6

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 2.0 - 2.1 using conc. HCl

↓

Add IPA, dodecylsodium sulphate
(40 mg for a 20 ml sample)

↓

Pass sample (10 ml) through a Sep Pak and discard effluent

↓

Wash Sep Pak with H_2O (5 ml) and discard

↓

Wash Sep Pak with a 35% MeOH:65% H_2O mixture (5 ml)
and discard

↓

Elute components of interest from the Sep Pak
with 100% methanol (5 ml)

↓

Make extract volume 10 ml by adding
0.2% H_3PO_4, pH 7.25

↓

Inject

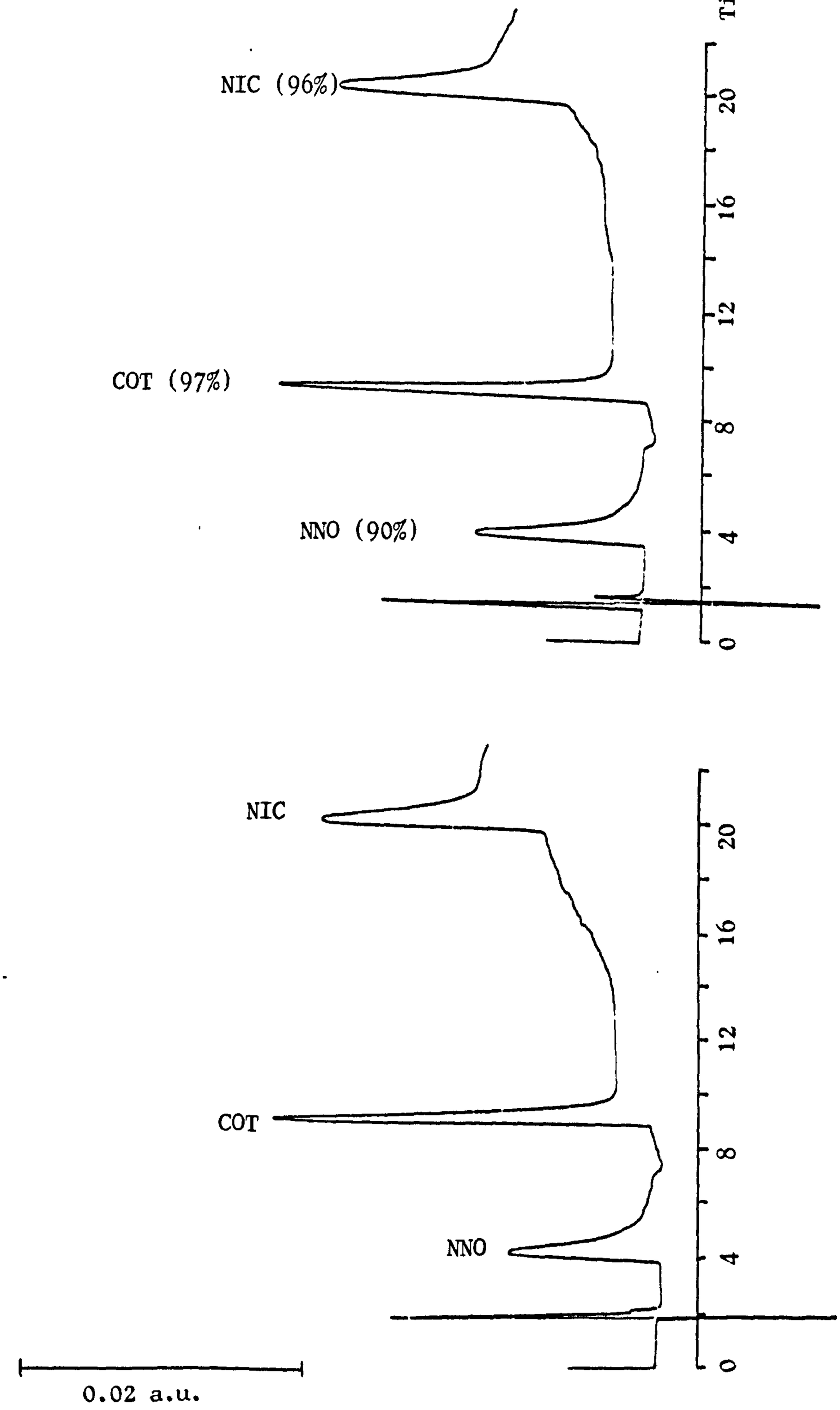


Figure 4.13: Extraction Procedure 6 (figure 4.12) applied to a standard mixture (10 μg ml^{-1}) in H_2O

Parameters: see figure 4.2 except for Gradient programme #5, 3 min initial hold at 24%B, 24% → 56%B over 14 mins. Flow Rate: 1.5 ml min^{-1}

Figure 4.14: Extraction Procedure 6 (figure 4.12) applied to a urine sample (female, non-smoker)
Parameters: see figure 4.5

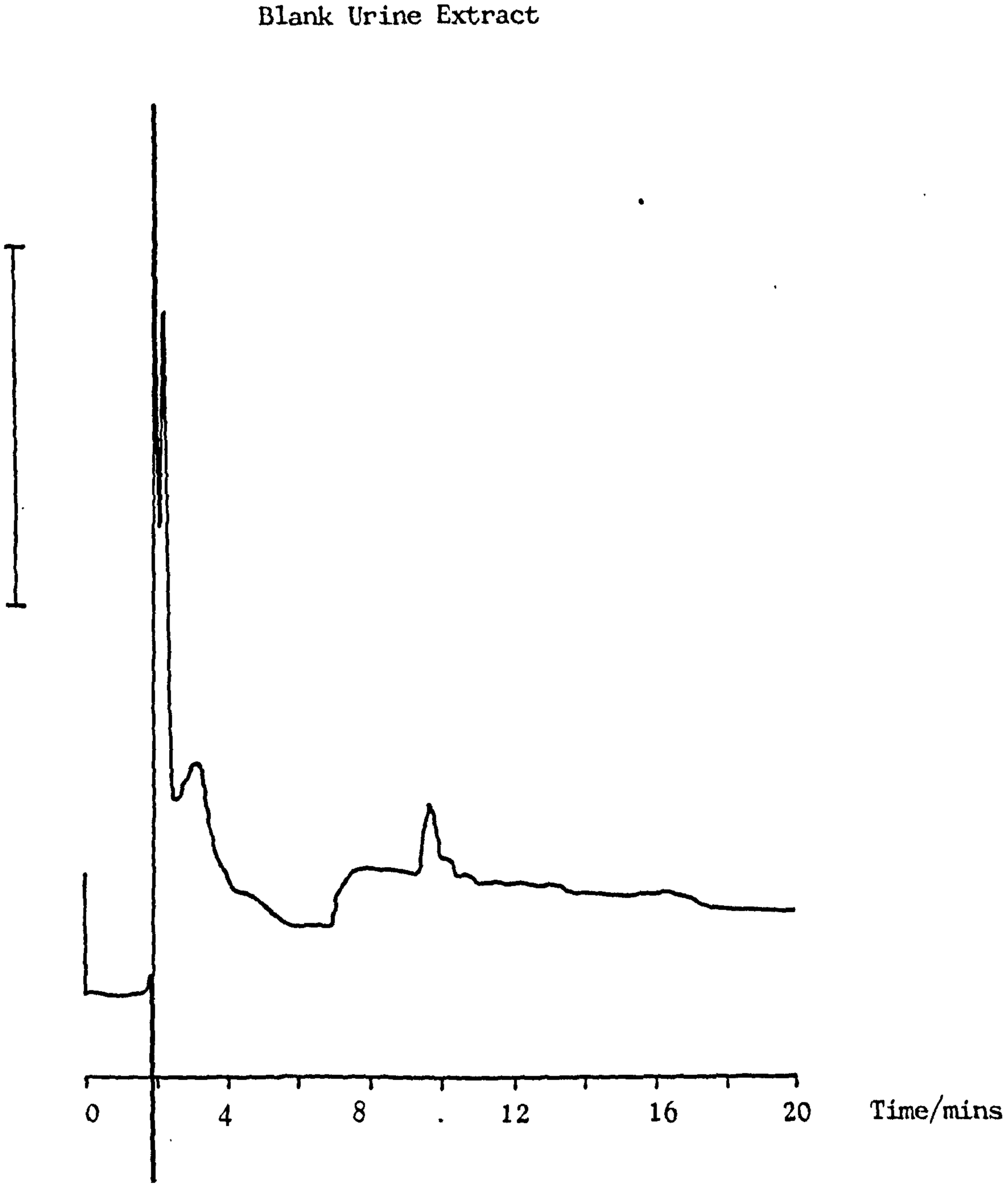


Figure 4.15: Extraction Procedure 6 (figure 4.12) applied to urine samples

Parameters: see figure 4.13

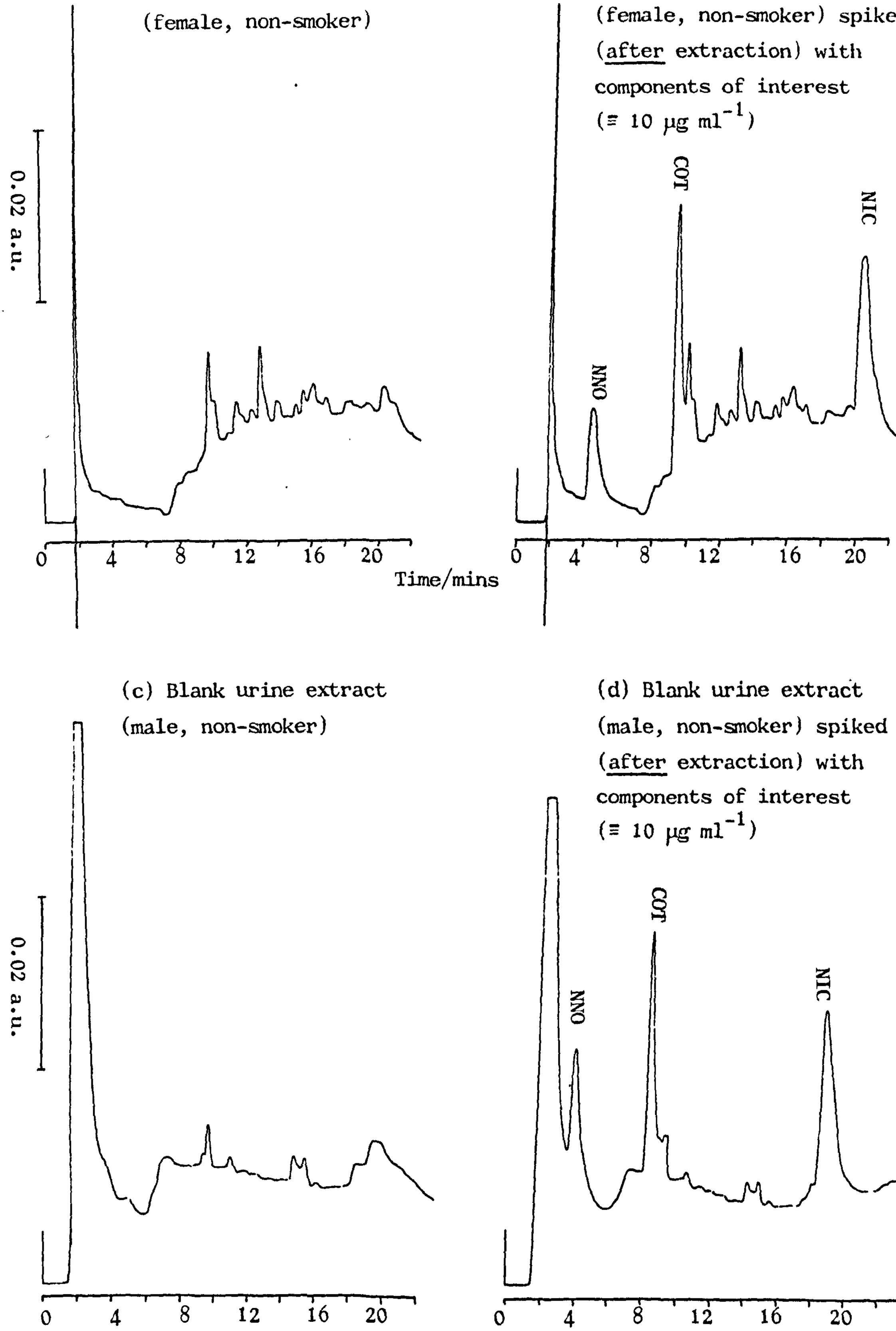
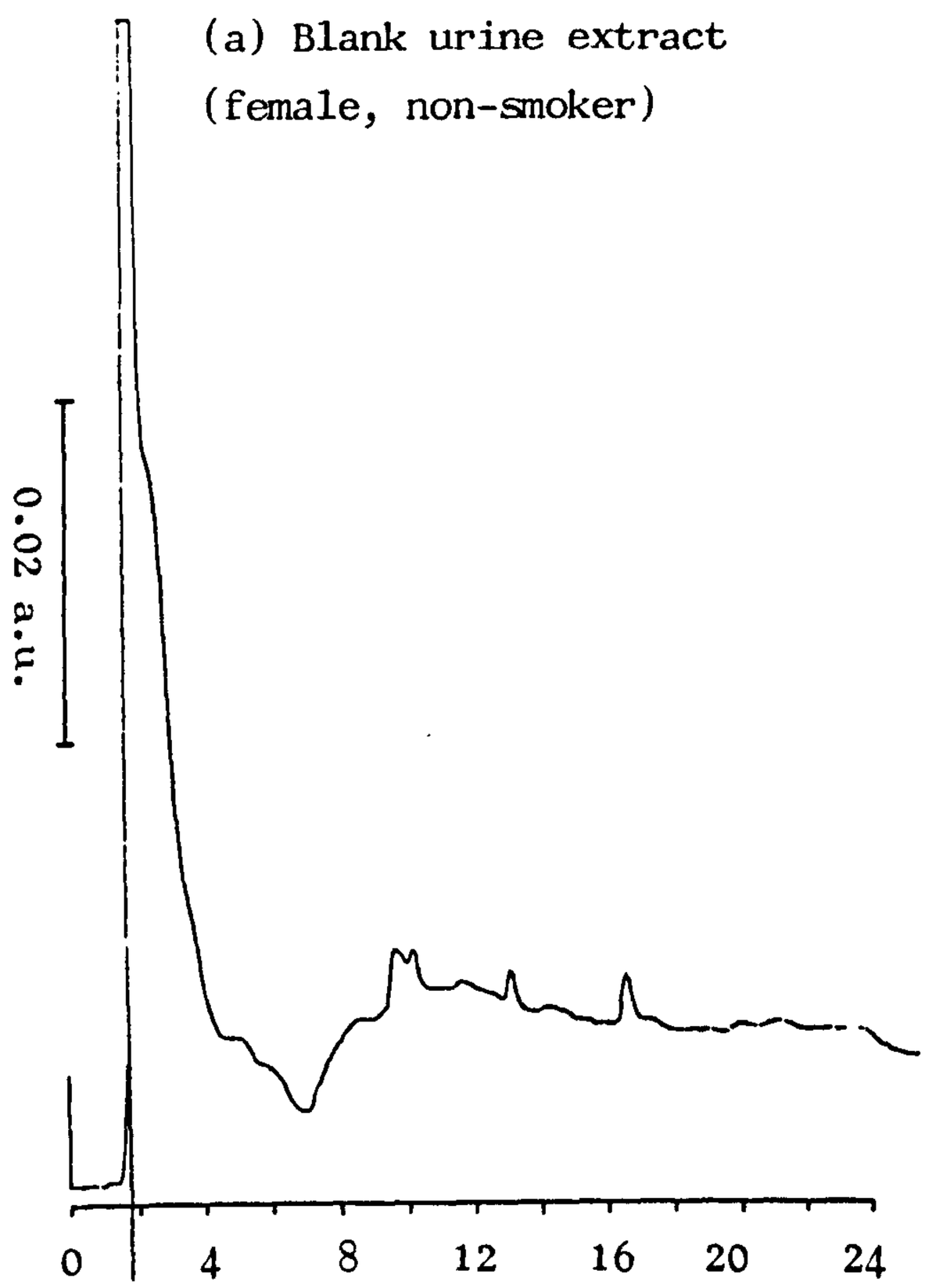
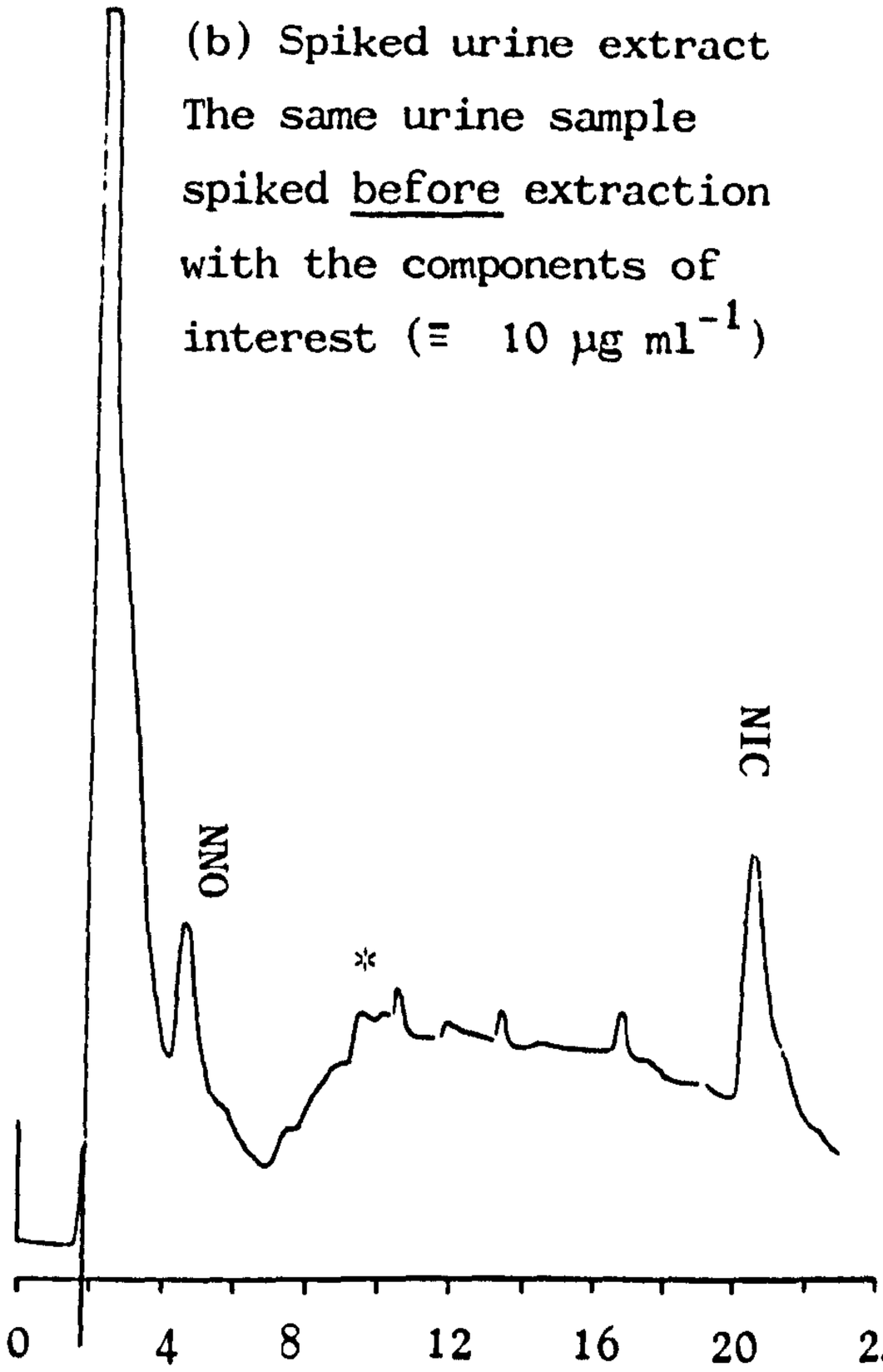
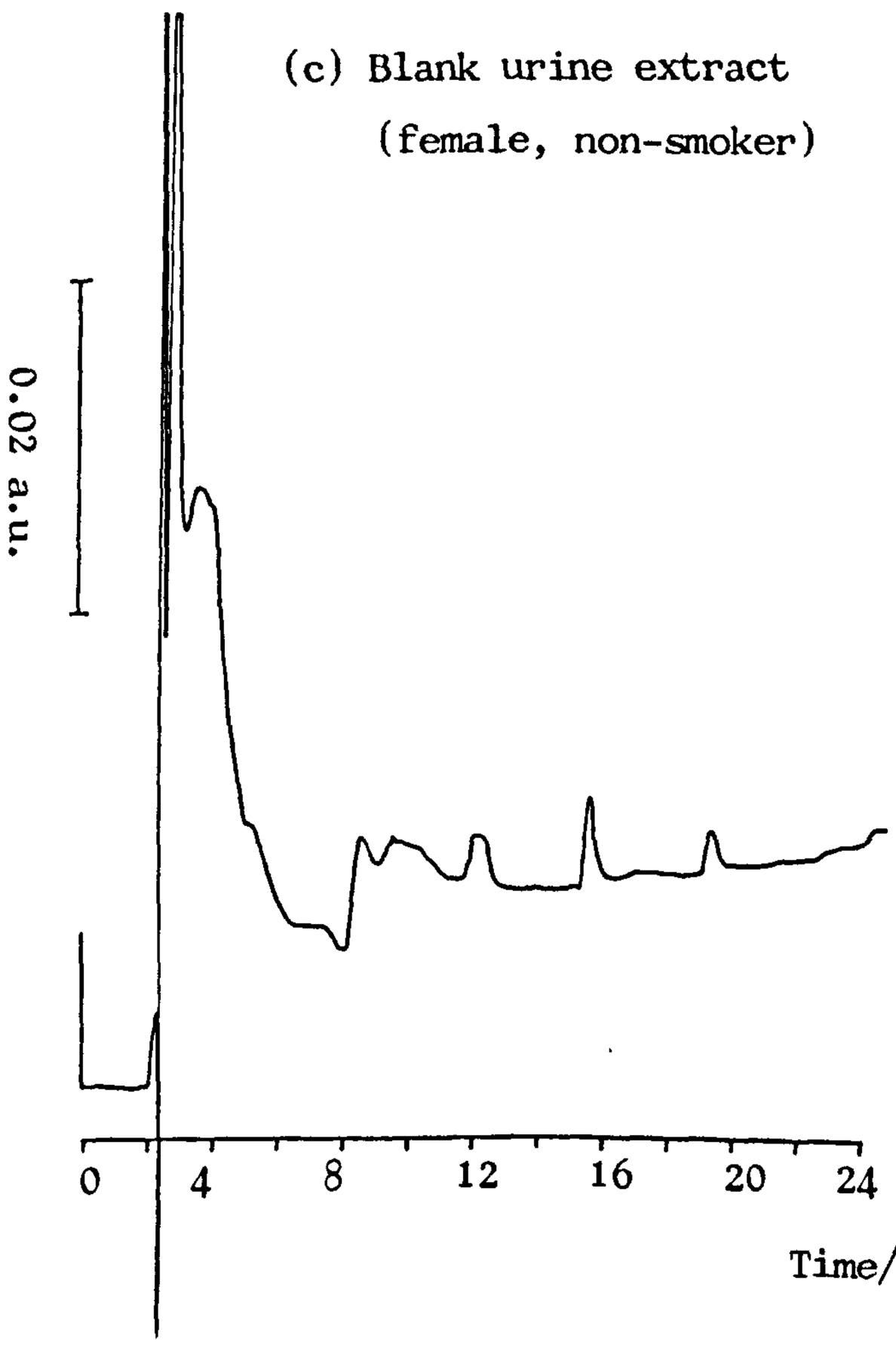


Figure 4.16: Extraction Procedure 6 (figure 4.12) applied to blank and spiked urine samples. **Parameters:** see figure 4.13







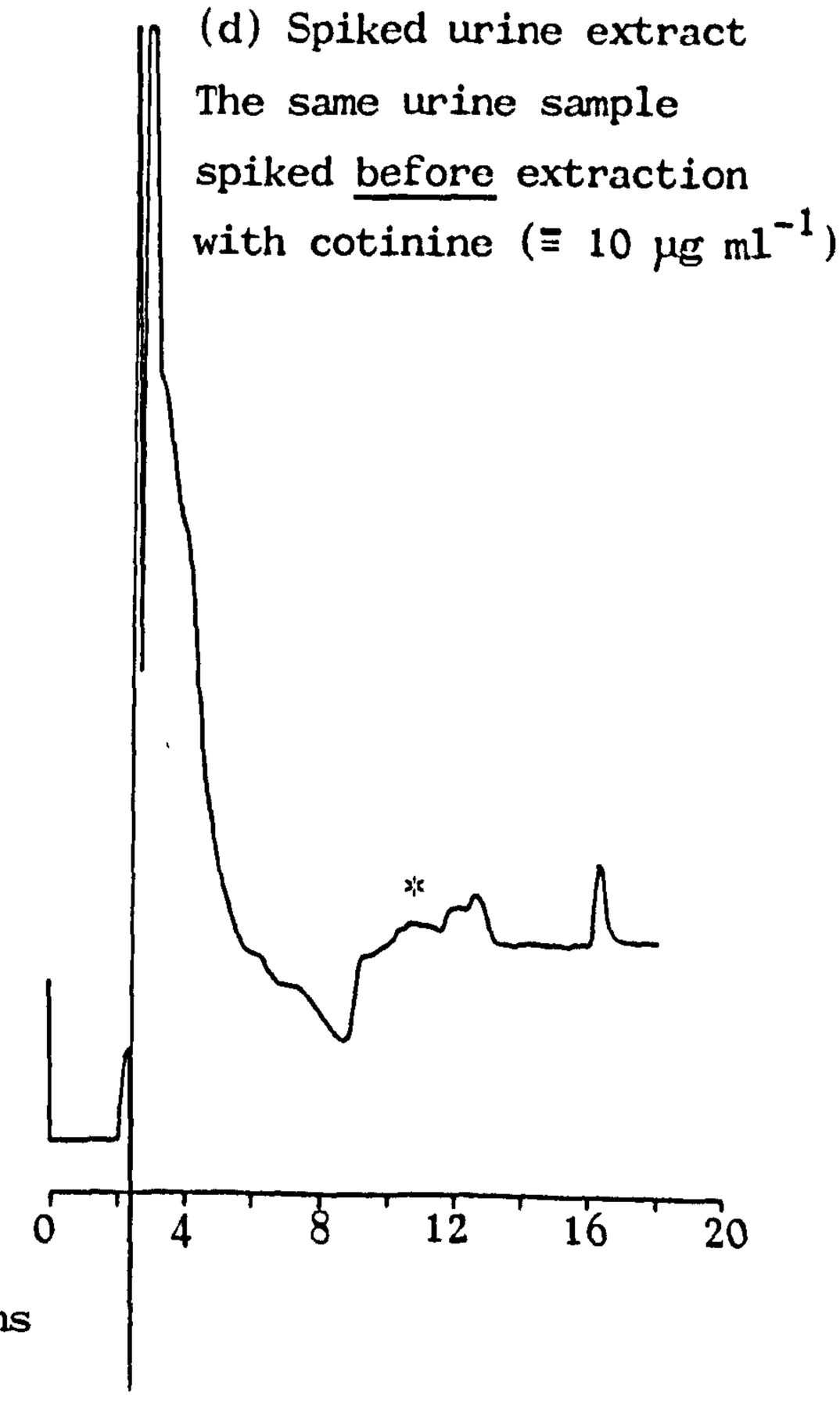
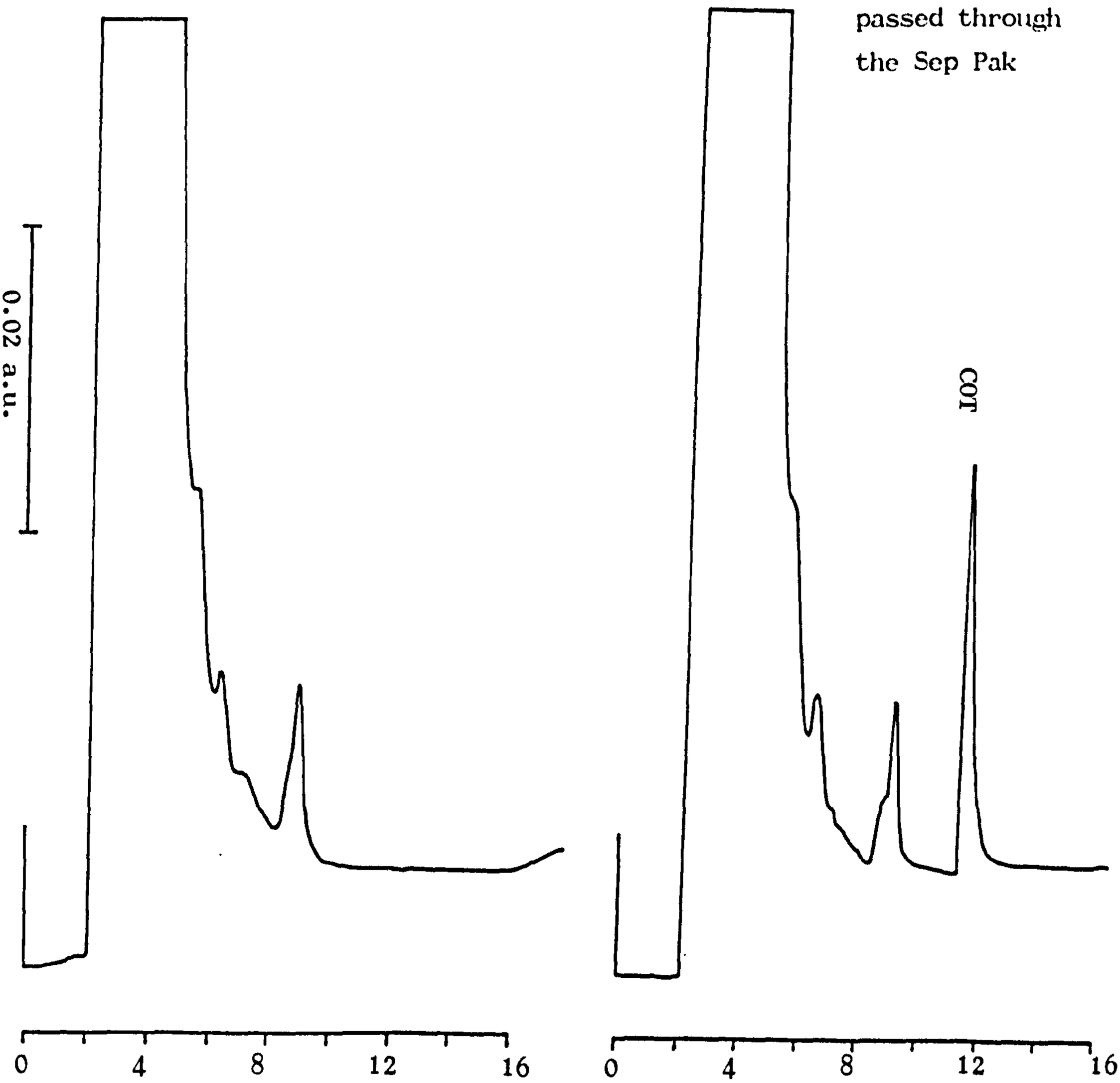


Figure 4.17: Chromatograms showing an intermediate step in extraction procedure 6 (figure 4.12) when applied to a blank and spiked urine sample (female, non-smoker).
Parameters: see figure 4.2 except for Flow Rate: 1.1 ml min^{-1}
Gradient programme #5, 3 min initial hold at 24%B, 24% → 54%B over 14 mins.

(a) Blank Urine. Effluent when the urine sample is initially passed through the Sep Pak

(b) Spiked Urine. Effluent when the same urine sample, spiked with cotinine (≡ 10 μg ml^{-1}) is initially passed through the Sep Pak



form a dedecylsulphate ion pair.

Simple variations in extraction procedure 6, figure 4.12, were examined, such as the use of excess dodecylsodium sulphate, the addition of the IPA before the pH was adjusted to 2.0, and the substitution of HCl, used to adjust the pH to 2.0, by H_3PO_4. Cotinine was not recovered, being lost in all cases, when the urine sample was passed through the Sep Pak initially.

In addition to the extraction at pH 2.0-2.1 with the IPA added, extractions of blank and spiked urine samples and a cotinine standard (10 $\mu g\ ml^{-1}$) in water were also carried out where the pH of the sample was adjusted to 1.0-1.1, 3.0-3.1 and 5.0-5.1 before the IPA was added and the remaining isolation steps in extraction scheme 6, figure 4.12, followed. Extractions at both pH 1.0 and pH 3.0 showed that cotinine was again lost when the spiked urine sample was passed through the Sep Pak initially. When the pH of the sample was adjusted to 5.0-5.1, cotinine was not lost when the sample was passed through the Sep Pak initially but when the Sep Pak was washed with a 35% methanol:65% water mixture. At pH 5.0-5.1 cotinine is not protonated and therefore does not form a dodecylsulphate ion pair, hence it is not possible to wash the Sep Pak with methanol: water mixtures without analyte loss.

Following extraction procedure 6, figure 4.12, but adjusting the pH to 7.0 and not 2.0, it was verified, using a standard mixture (10 $\mu g\ ml^{-1}$), that a methanol:water mixture (even in the ratio 30:70) could not be used to wash the Sep Pak as it resulted in the complete loss of both nicotine-1'-N-oxide and cotinine. In order to include this step in the clean-up scheme, not only must

the IPA be present but the pH must be adjusted so that the components of interest are protonated and so can form ion pairs.

Extraction of a standard mixture in water again following extraction procedure 6, figure 4.12, but on this occasion without the addition of the ion-pairing agent, resulted, as expected, in the complete loss of nicotine and nicotine-1'-N-oxide when the sample was passed through the Sep Pak. 92% of the cotinine present originally was unretained by the Sep Pak and therefore lost with the nicotine-1'-N-oxide and nicotine. The retained cotinine was lost during later isolation steps when the Sep Pak was washed with water and a methanol:water mixture (35:65).

Blank and spiked urine samples were also examined in each of the above experiments; the same end results as in the experiments with the standards were obtained. However, due to the complexity of chromatographs showing the effluent from the Sep Pak after each stage in the extraction schemes, it was not always possible to confirm the exact step(s) at which the components of interest were lost.

From extraction procedure 1, figure 4.1, where the sample is adjusted to pH 7.0, it has been shown that cotinine can be extracted successfully. Therefore, when extraction scheme 6 is applied to a urine sample, the initial loss from the Sep Pak can be collected, the pH readjusted to 7.0 (with e.g. NaOH), and then the isolation steps reported in extraction scheme 1 applied; this is summarised in figure 4.18. It was hoped that by the addition of these steps, cotinine could be successfully recovered and this is shown to be true, see figure 4.19.

Figure 4.18: Extraction Procedure 7

Sample (20 ml) : urine or standard mixture in H_2O (10 $\mu g\ ml^{-1}$)

↓

Adjust pH to 2.0 - 2.1 using conc. HCl

↓

Add the IPA dodecylsodium sulphate
(40 mg for a 20 ml sample)

↓

Pass sample (10 ml) through a Sep Pak and collect

↓

Adjust pH to 7.0 with NaOH

↓

Pass through another Sep Pak and discard effluent

↓

Wash Sep Pak with H_2O (5 ml) and discard

↓

Elute components of interest from Sep Pak
with 100% methanol (5 ml)

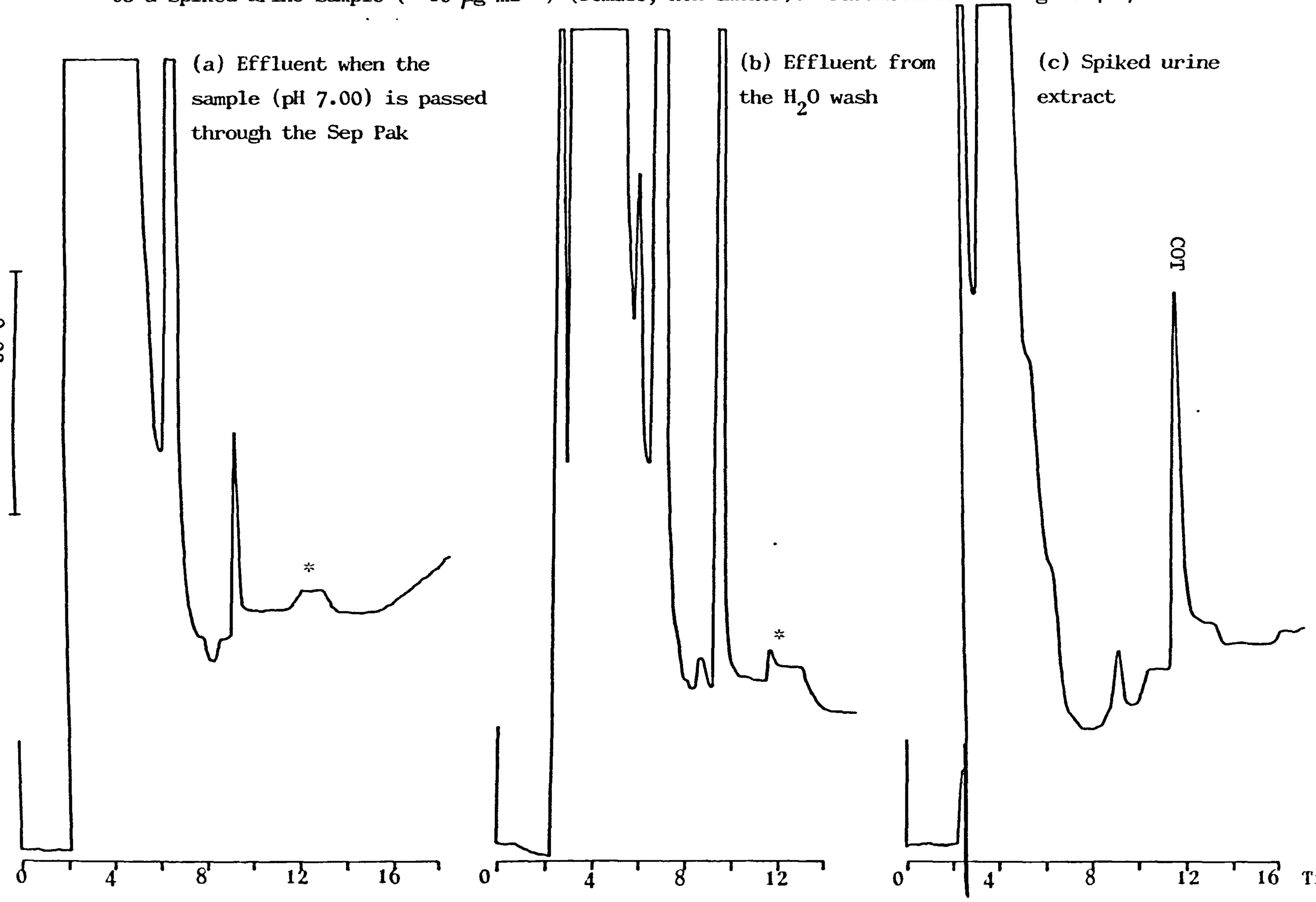
↓

Make extract volume 10 ml by adding
0.2% H_3PO_4, pH 7.25

↓

Inject

Figure 4.19: Chromatograms showing intermediate steps and final extract when extraction procedure 7 is applied to a spiked urine sample (≡ 10 μg ml^{-1}) (female, non-smoker). **Parameters:** see figure 4.17



For the analysis of smokers' samples or indeed samples spiked with all three components of interest before extraction, the final overall scheme would obviously involve the combination of the extract containing nicotine and nicotine-1'-N-oxide and that containing cotinine, before injection, i.e. a combination of extraction procedures 6 and 7 as shown in figure 4.20, extraction procedure 8.

In order to maximize the effectiveness of extraction procedure 8, figure 4.20, eluting the components of interest with a pH 2.0 solution, in place of neat methanol, was considered. This made no significant difference to the cleaned-up sample extracts and was abandoned. The pH 2.0 solution, if used, would not be compatible with the column and mobile phase used for the analysis, so making another pH adjustment necessary.

It would be possible to substitute a methanol:water, 40:60, Sep Pak wash in place of the 35:65 one already in use; as shown in figure 4.10, nicotine and nicotine-1'-N-oxide are not lost in either case. Again, however, the inclusion of a 40% methanol:60% water wash did not result in any improvement in the clean-up of the samples.

Interfering peaks have not so far been examined. Caffeine is a substance known to be present in urine, having been identified by GC analysis.[182] HPLC analysis of a caffeine standard (10 $\mu g\ ml^{-1}$) in water showed that it co-eluted with cotinine, see figure 4.21. Fortunately, when extraction procedure 6 was applied to the caffeine standard, examination of the effluent from the Sep Pak after each isolation step revealed that caffeine was lost when the Sep Pak was washed with a methanol:water (35:65) mixture.

Figure 4.20: Extraction Procedure 8

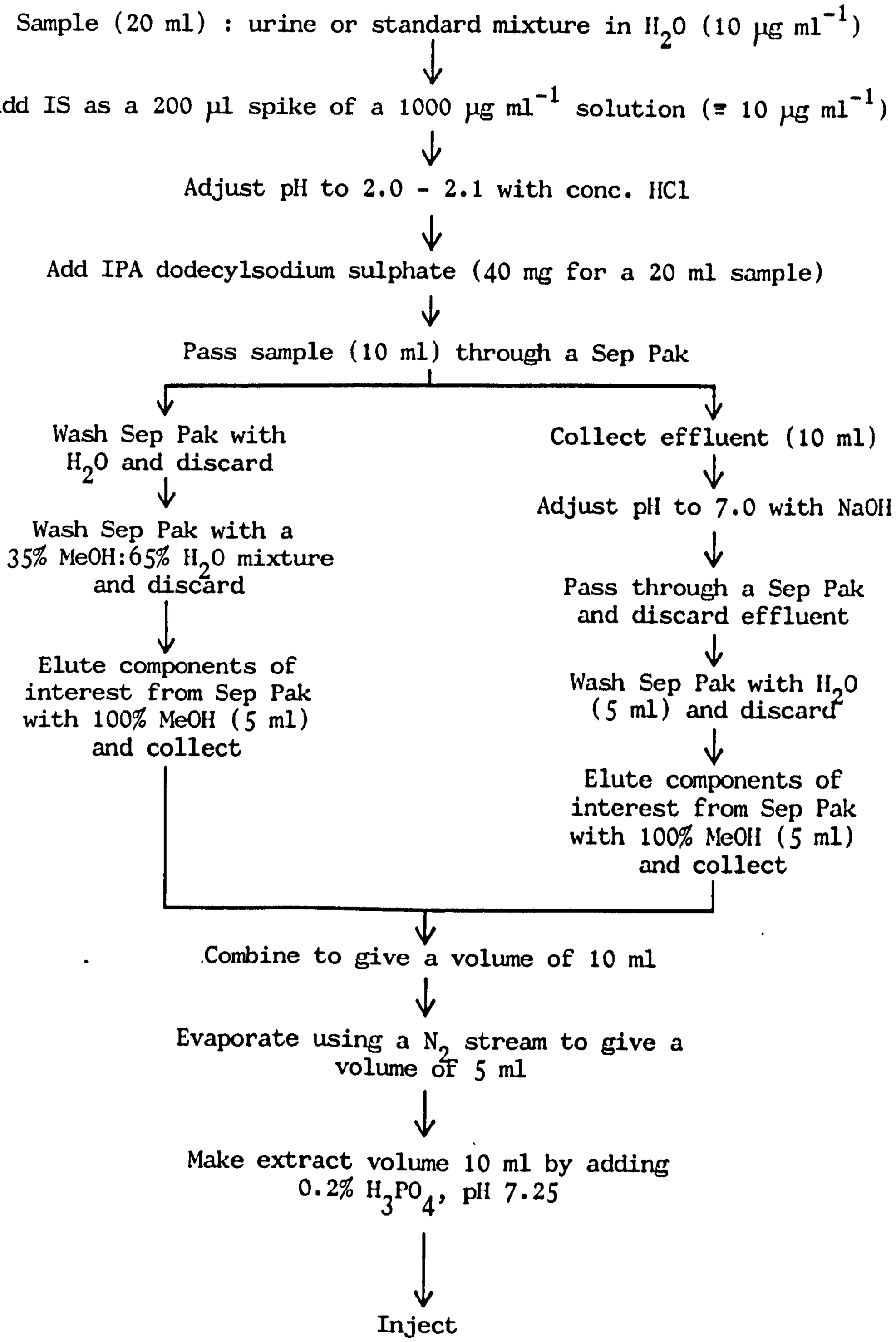
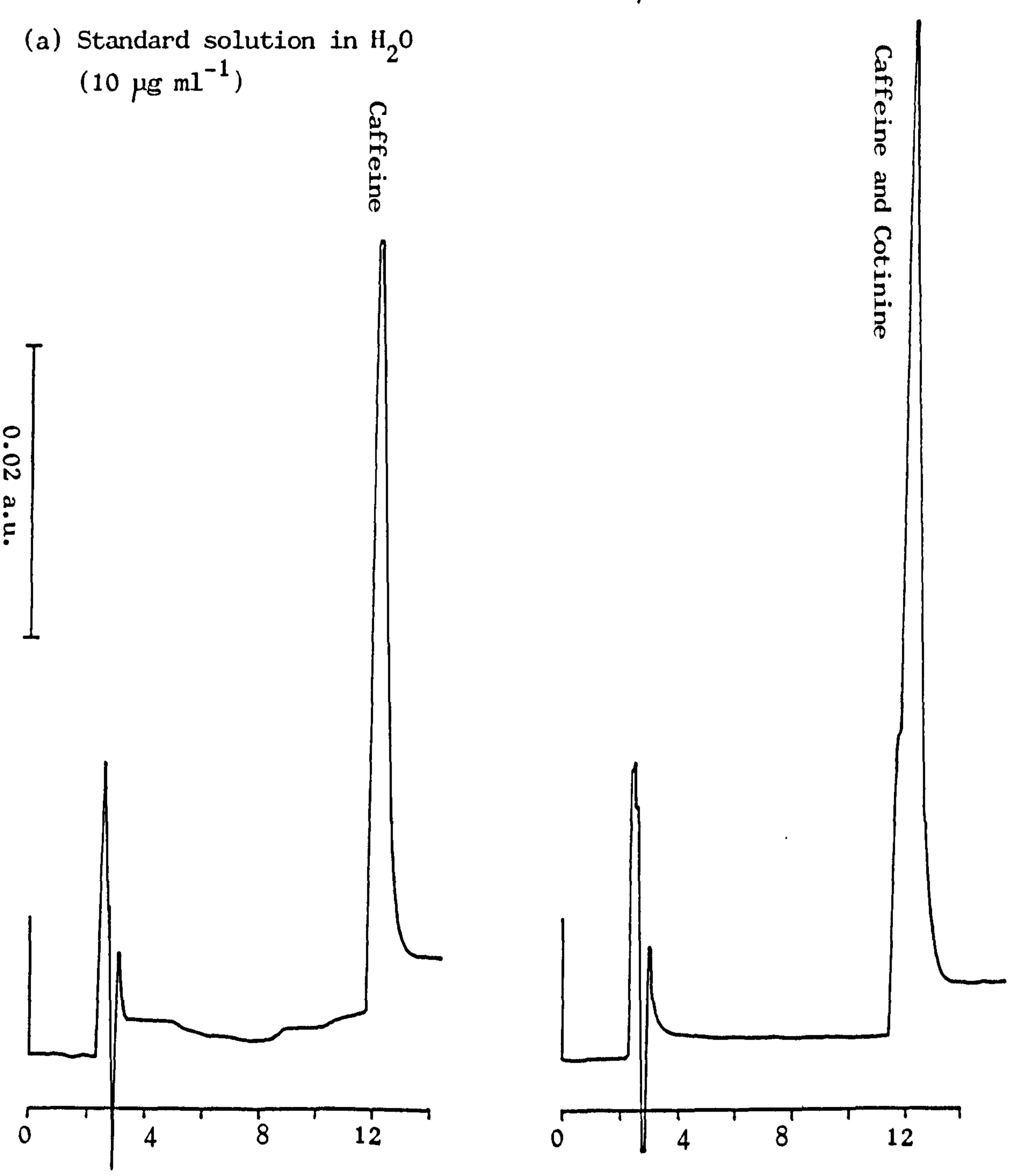


Figure 4.21: Chromatograms showing a caffeine standard (10 $\mu g\ ml^{-1}$) and its potential interference with cotinine

Parameters: see figure 4.17



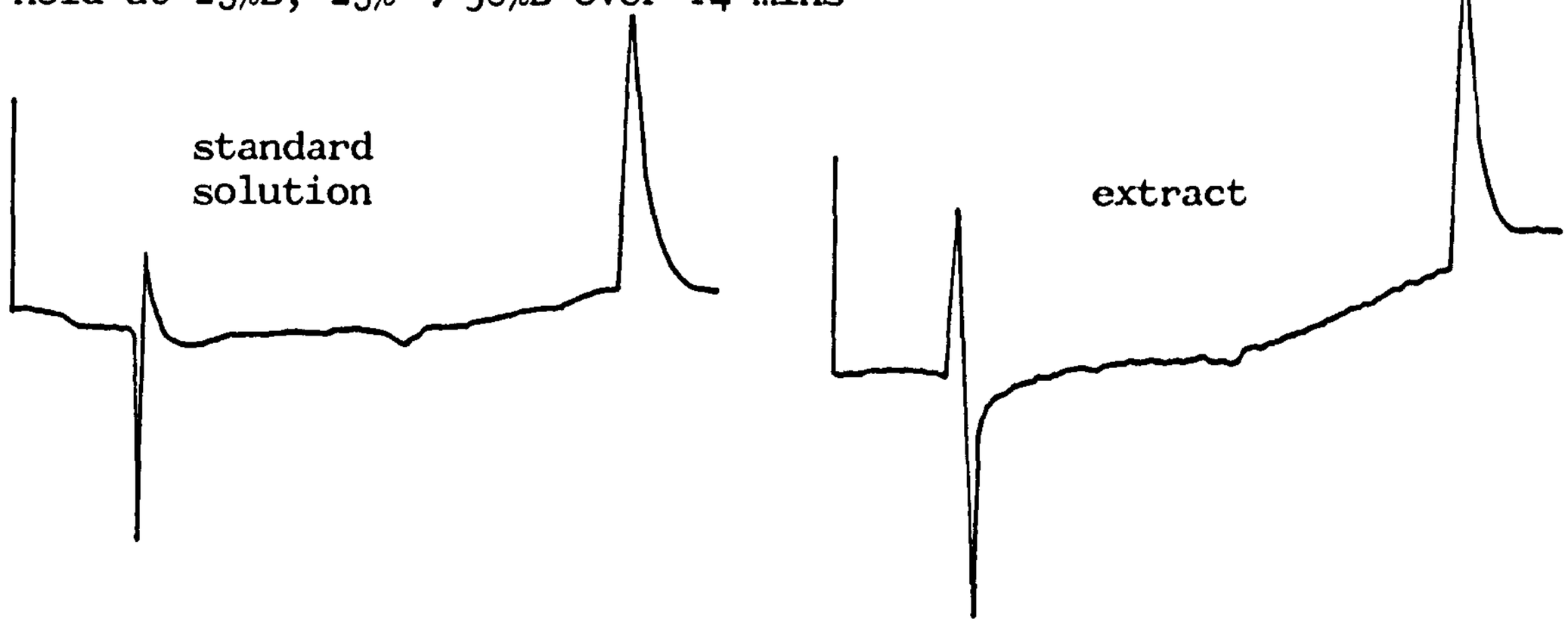
4.8.2 Extraction procedures applied to the potential internal standards

Before further investigation of the clean-up/extraction of blank and spiked urine samples and finally smokers' urine samples, extraction procedure 6, figure 4.12, was applied to solutions of the potential internal standards selected in the previous chapter, section 2.10, on the basis of their stability and retention times. Each of the standard solutions (10 $\mu g\ ml^{-1}$) were successfully extracted with the following recovery rates: β-nicotyrine, (87%); N'-acetyl-nornicotine (94%); and 2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine (100%), as shown in figure 4.22. When the internal standards were added to a blank urine sample (≡ 10 $\mu g\ ml^{-1}$) and extracted, see figure 4.23, the 2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine and β-nicotyrine were extracted with excellent recovery rates of 100% and 91% respectively. However, only 40% N'-acetyl nornicotine was recovered and on investigation of the effluent from the Sep Pak after each stage in the scheme, N' acetyl nornicotine was found to have been lost during the initial step when the urine sample was first passed through the Sep Pak (∿ 41%), as shown in figure 4.24, and also when the Sep Pak was washed with water (∿ 10%), see figure 4.25. N' acetyl nornicotine may also have been lost when the Sep Pak was washed with a methanol:water (35:65) mixture, however the complexity of the chromatogram of the effluent made identification of the N' acetyl nornicotine peak impossible. Therefore the use of extraction procedure 6 eliminated N' acetyl nornicotine as an internal standard and as already reported in section 2.10, β-nicotyrine was also discarded due to the instability of

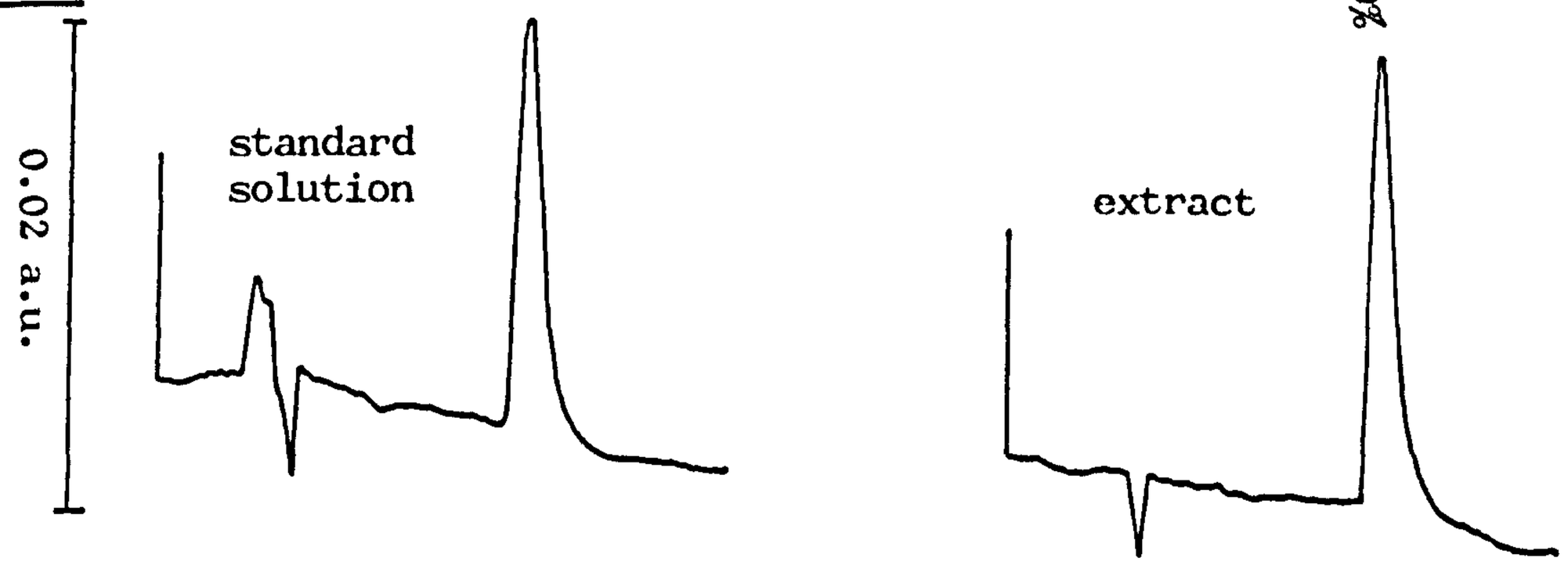
Figure 4.22: Extraction Procedure 6 (figure 4.12) applied to potential internal standard solution in H_2O (10 $\mu g\ ml^{-1}$)

Parameters: see figure 4.2 except for Flow Rate: 1.1 ml min^{-1} and as indicated below.

(a) N' Acetyl-S-(-) nornicotine. Eluent: gradient programme #5, 3 min initial hold at 23%B, 23% → 58%B over 14 mins



(b) 2 methyl-6-(3-pyridyl)tetrahydro-(1,2) oxazine
Eluent: isocratic at 60%B



(c) β-nicotyrine. Eluent: isocratic at 60%B

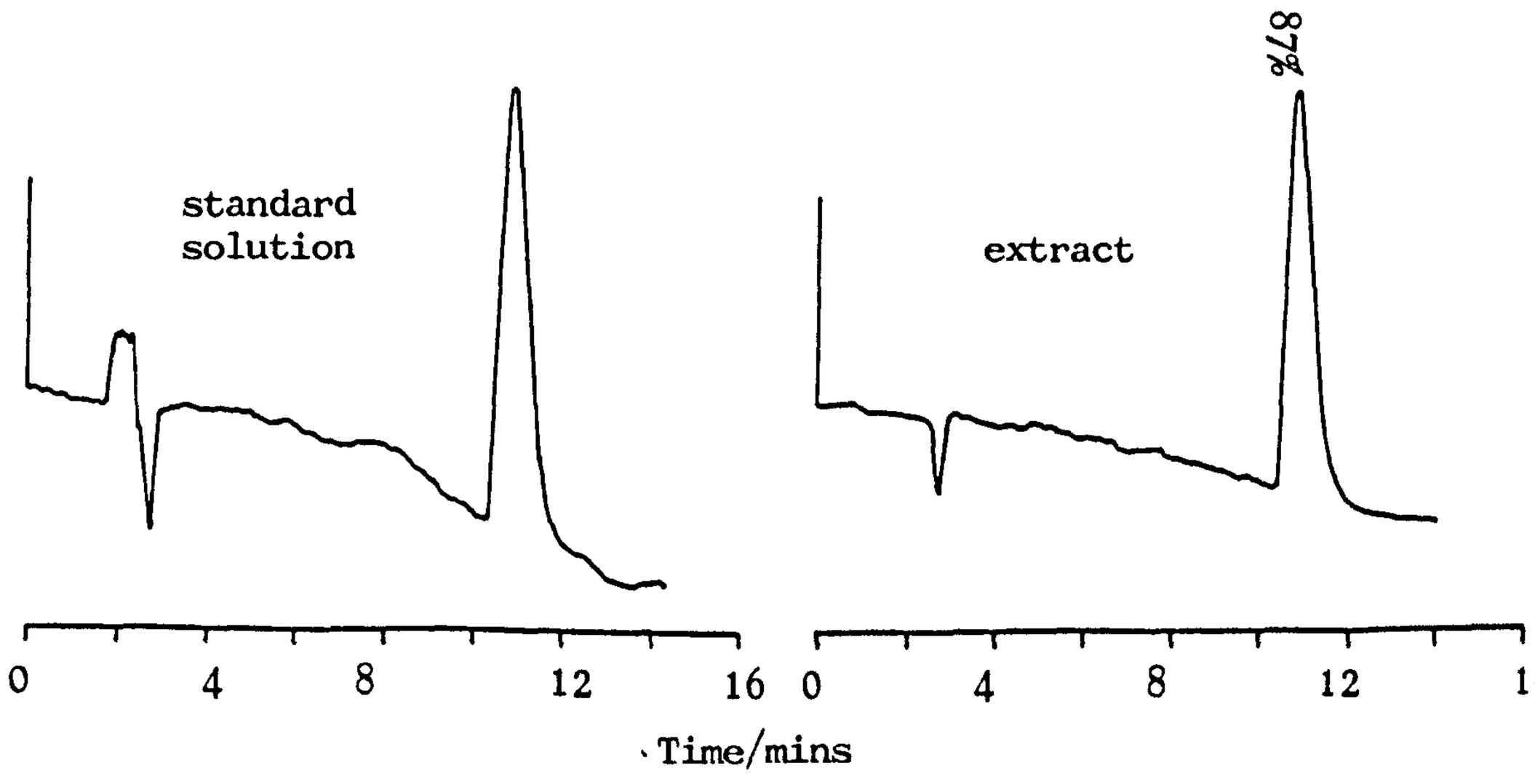


Figure 4.23: Extraction Procedure 6 (figure 4.12) applied to a urine sample (female, non-smoker), blank and spiked with the potential internal standards. **Parameters:** see figure 4.2 except for: Flow Rate: 1.1 ml min^{-1}. Gradient programme #5, 4 min initial hold at 23%B, 23% → 58%B over 14 mins

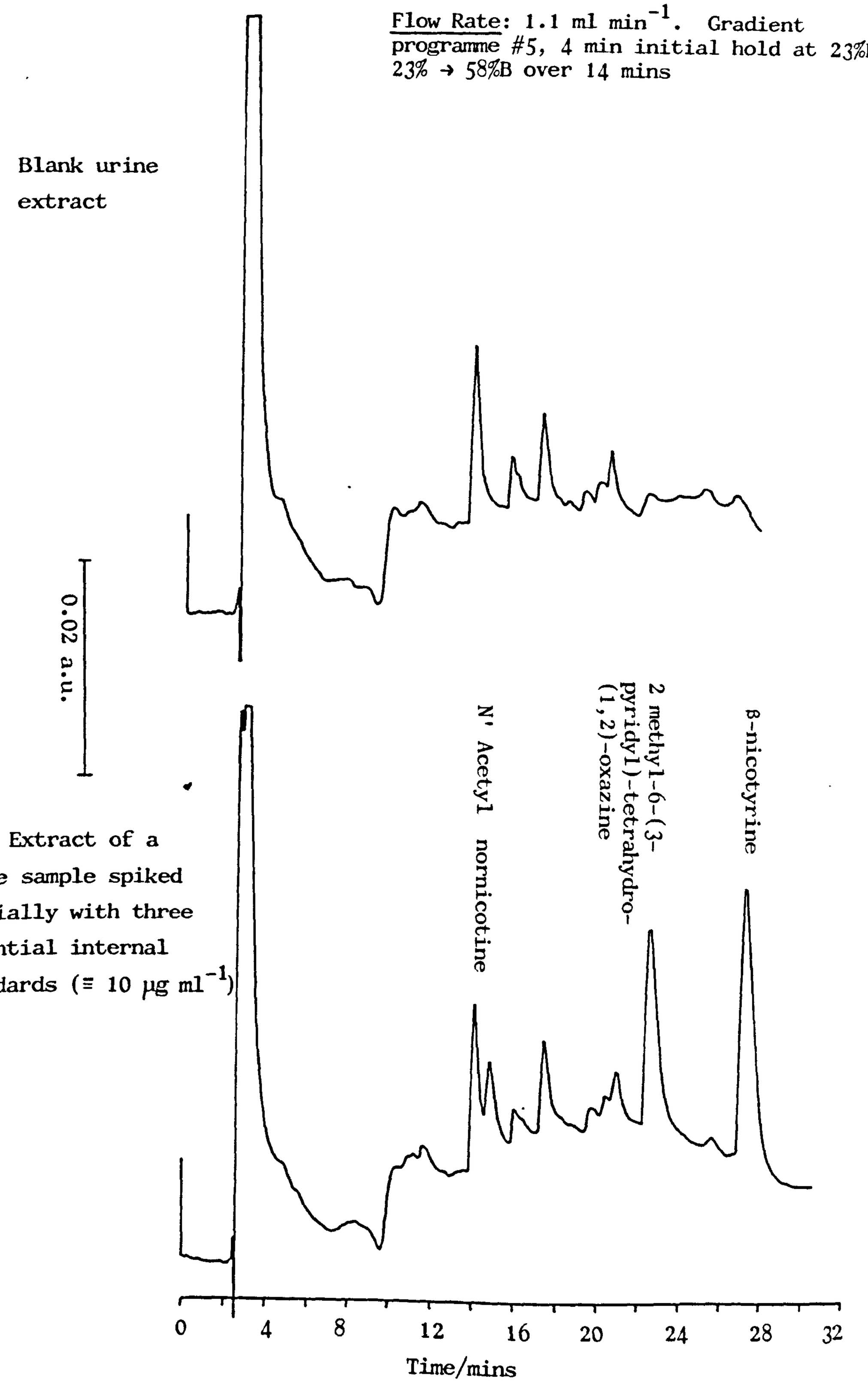


Figure 4.24: Chromatograms showing an intermediate step in Extraction Procedure 6 (figure 4.12) when applied to a urine sample spiked initially with N' Acetyl nornicotine (≡ 10 μg ml^{-1}). **Parameters:** see figure 4.23

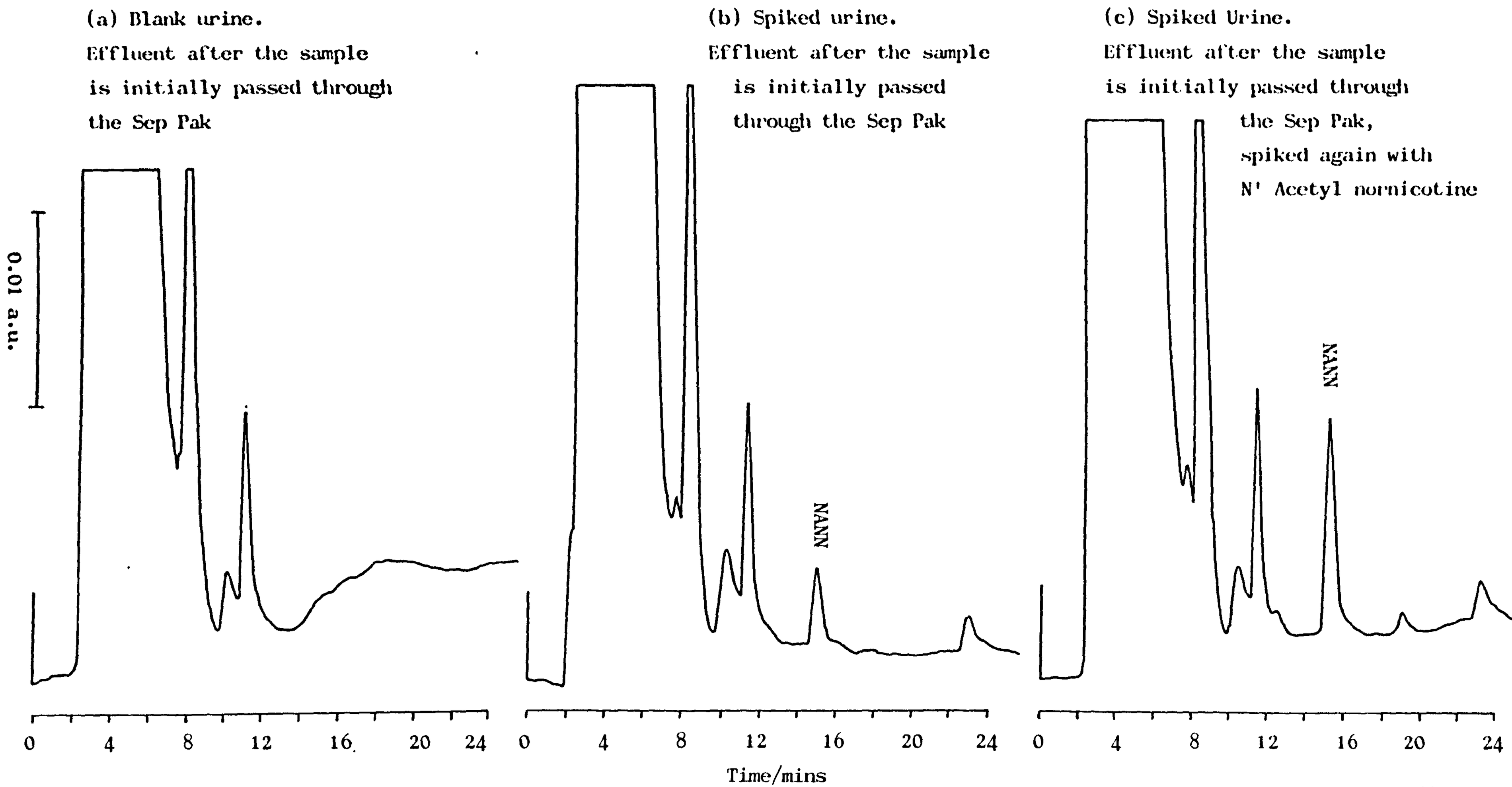
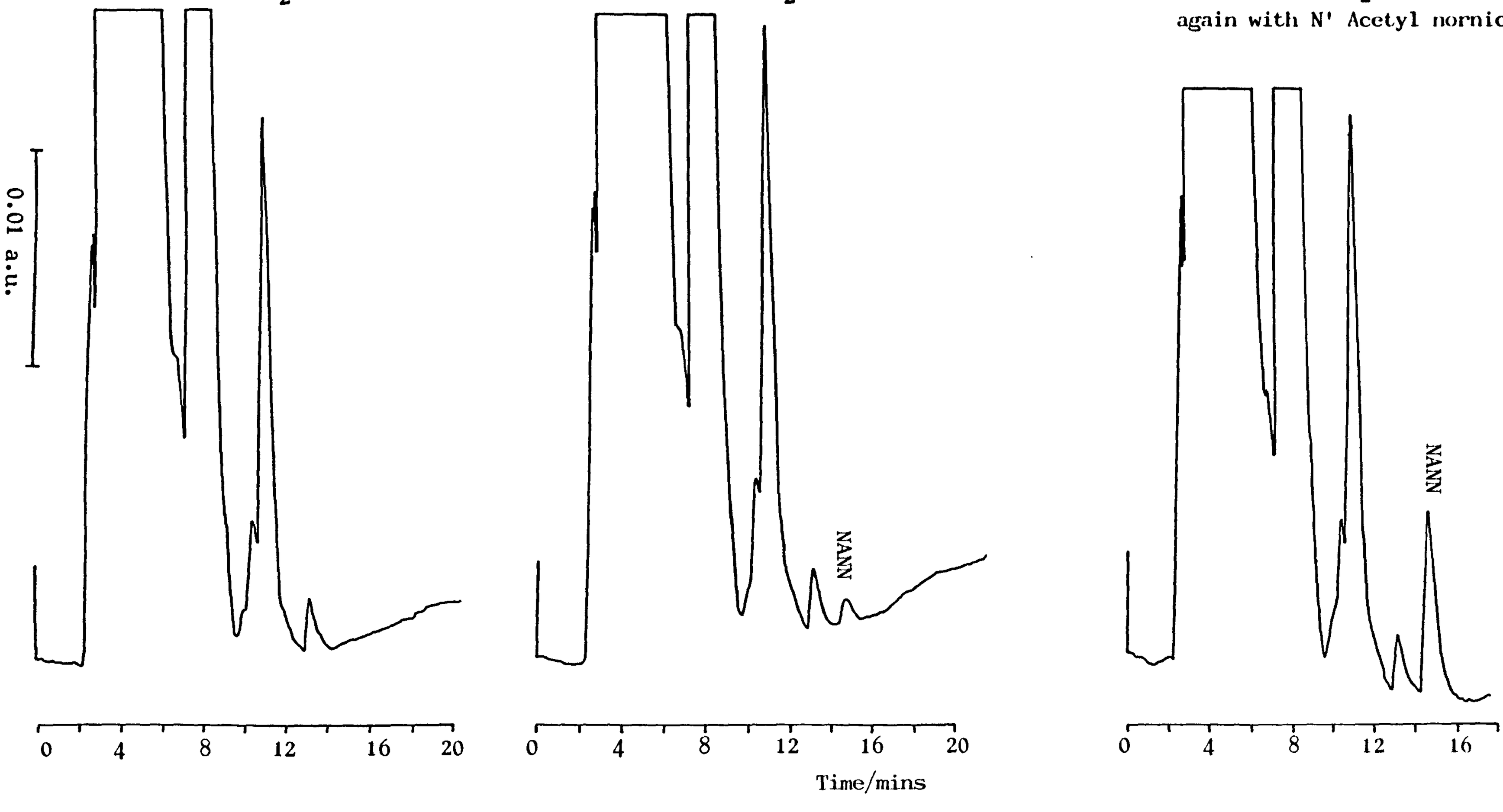


Figure 4.25: Chromatograms showing an intermediate step in Extraction Procedure 6 (figure 4.12) when applied to a urine sample spiked initially with N' Acetyl nornicotine (≡ 10 μg ml^{-1}). **Parameters:** see figure 4.23



its solution in water. In all the preliminary experiments the IS was added as a spike, from a 1000 μg ml^{-1} solution in H_2O, (≡ 10 μg ml^{-1}) to the sample. If a concentration step is included the initial concentration of the IS must be altered accordingly.

4.8.3 The Extraction of 3' Hydroxycotinine

A 3' hydroxycotinine standard solution can be extracted, again following extraction procedure 6, figure 4.12, with a recovery value of 67%, 13% being lost when the solution was passed through the Sep Pak, as shown in figure 4.26. Extractions of blank and spiked urine (≡ 10 μg ml^{-1} 3' hydroxycotinine) revealed that 3' hydroxycotinine was lost during the extraction process, as shown in figure 4.27. Unlike cotinine, it was impossible, on this occasion, to confirm at what stage(s) 3' hydroxycotinine was lost. A contributory factor was the fact that 3' hydroxycotinine was eluted early in the chromatogram, not being strongly retained by the ODS column used in the analysis and hence the 3' hydroxycotinine peak was usually not resolved from the many other peaks, of no analytical interest, present in the effluent.

Having previously investigated the loss of cotinine from a spiked urine sample and achieved its subsequent recovery, accompanied by the satisfactory clean-up of the urine sample, using extraction procedure 7, figure 4.18, the same scheme was applied to a urine sample spiked with 3' hydroxycotinine, under the assumption that 3' hydroxycotinine would behave as in the case of cotinine. Indeed, when the extraction scheme was applied to both blank and spiked (≡ 10 μg ml^{-1} 3' hydroxycotinine) urine samples, 3' hydroxycotinine

Figure 4.26: Extraction Procedure 6 (figure 4.12) applied to a standard solution of 3' Hydroxycotinine in H_2O (10 μg ml^{-1}). **Parameters:** see figure 4.2 except for Flow Rate: 1.3 ml min^{-1}. Eluent: isocratic with 22%B

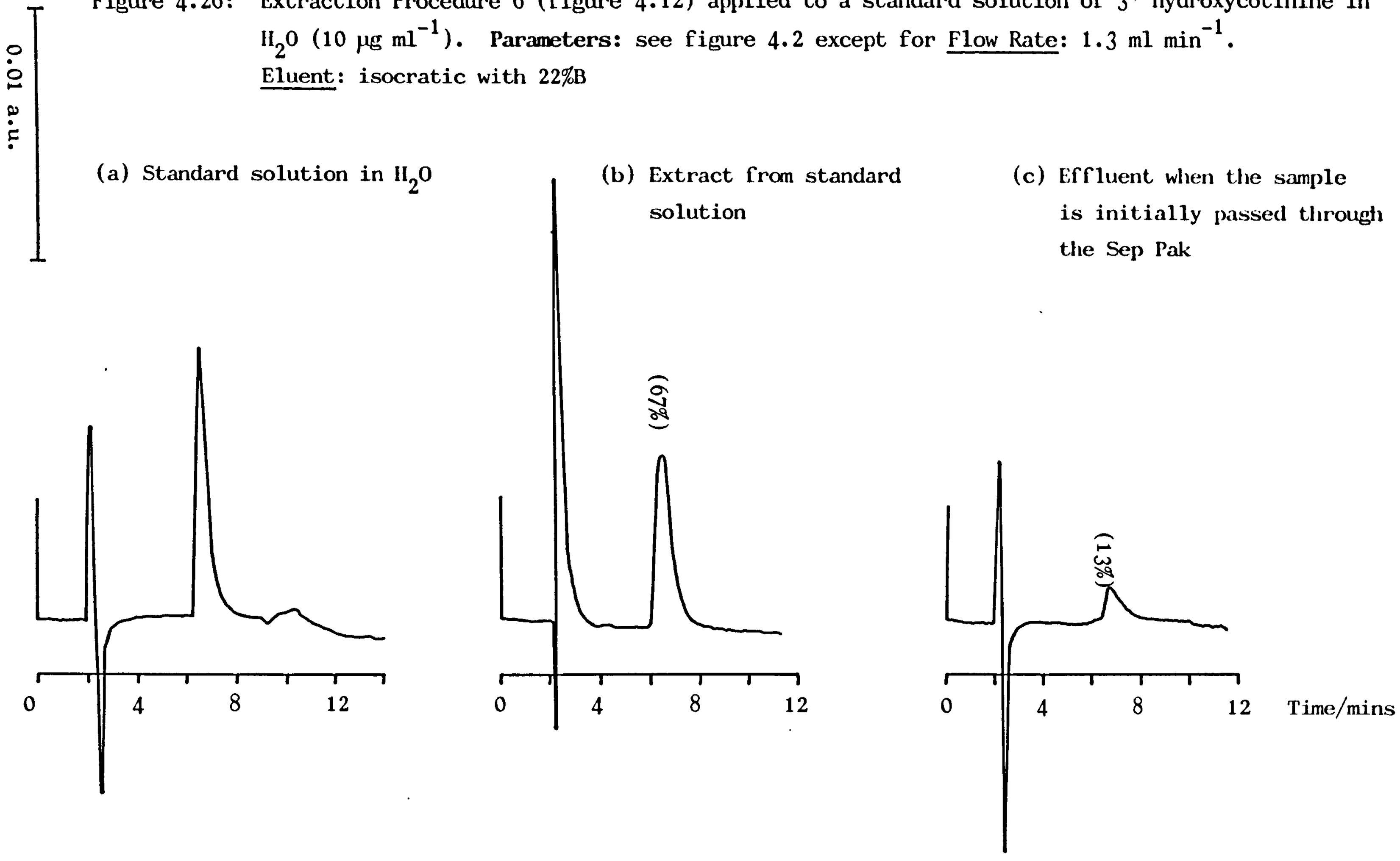
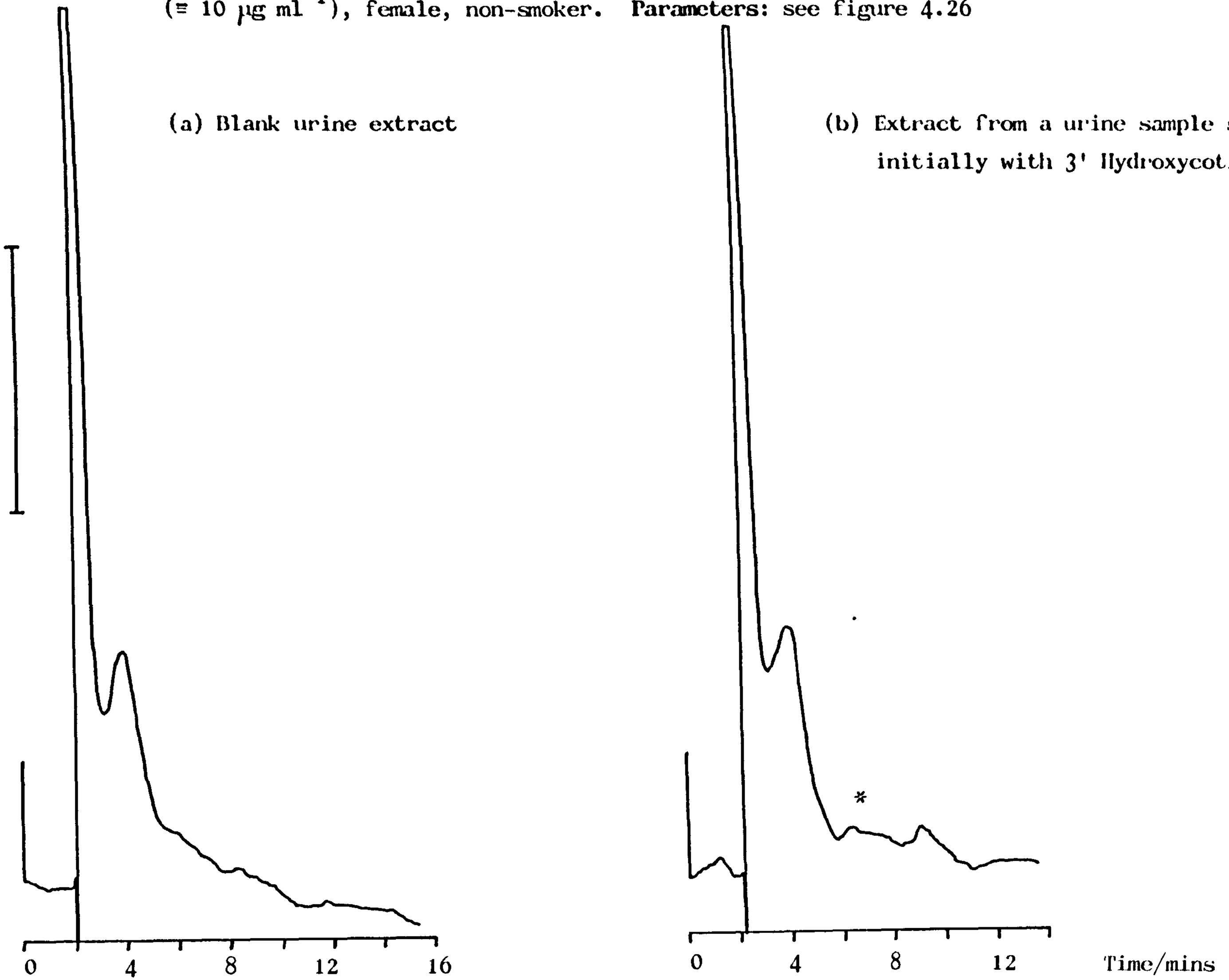


Figure 4.27: Extraction Procedure 6 (figure 4.12) applied to a urine sample, blank and spiked with 3' Hydroxycotinine (≡ 10 μg ml^{-1}), female, non-smoker. **Parameters:** see figure 4.26



was extracted, however an interfering peak was also present, see figure 4.28. On repeating the experiment with other urine samples, the unidentified interfering peak was also present, as shown in figure 4.29. In all the experiments carried out so far, the urine samples have been spiked with ≡ 10 μg ml^{-1} of the components of interest and so interference peaks may not at first be noticed. However, the levels of nicotine and its metabolites in smokers' samples were expected to be low and therefore any interference may impair or devalue the analysis.

Extraction procedure 8, figure 4.20, must be used if <u>all</u> four components of interest are to be successfully recovered from a urine sample. The additional steps incorporated in extraction procedure 8 unfortunately lead to more components, of no analytical interest, also being extracted. Indeed, a component which interfered with 3' hydroxycotinine was co-extracted as shown in figures 4.28 and 29. Since 3' hydroxycotinine had been included in the analysis, the gradient programme in use previously, e.g. figure 4.5, had to be altered, as a lower percentage methanol was required to resolve 3' hydroxycotinine and nicotine-1'-N-oxide and this also resulted in increased k' values for other components which had been eluted from the column with low k' values of between 0 and 2.

From figures 4.30 and 4.31 it was evident that there was a problem concerning the resolution of both nicotine-1'-N-oxide and 3' hydroxycotinine from other components which are eluted in the void volume and early in the chromatogram.

Figure 4.28: Extraction Procedure 7 applied to a urine sample, blank and spiked with 3' Hydroxycotinine (≡ 10 μg ml^{-1}) male, non-smoker. **Parameters:** see figure 4.2 except for Flow Rate: 1.0 ml min^{-1}. Gradient programme #6, 17% → 37%B over 15 mins

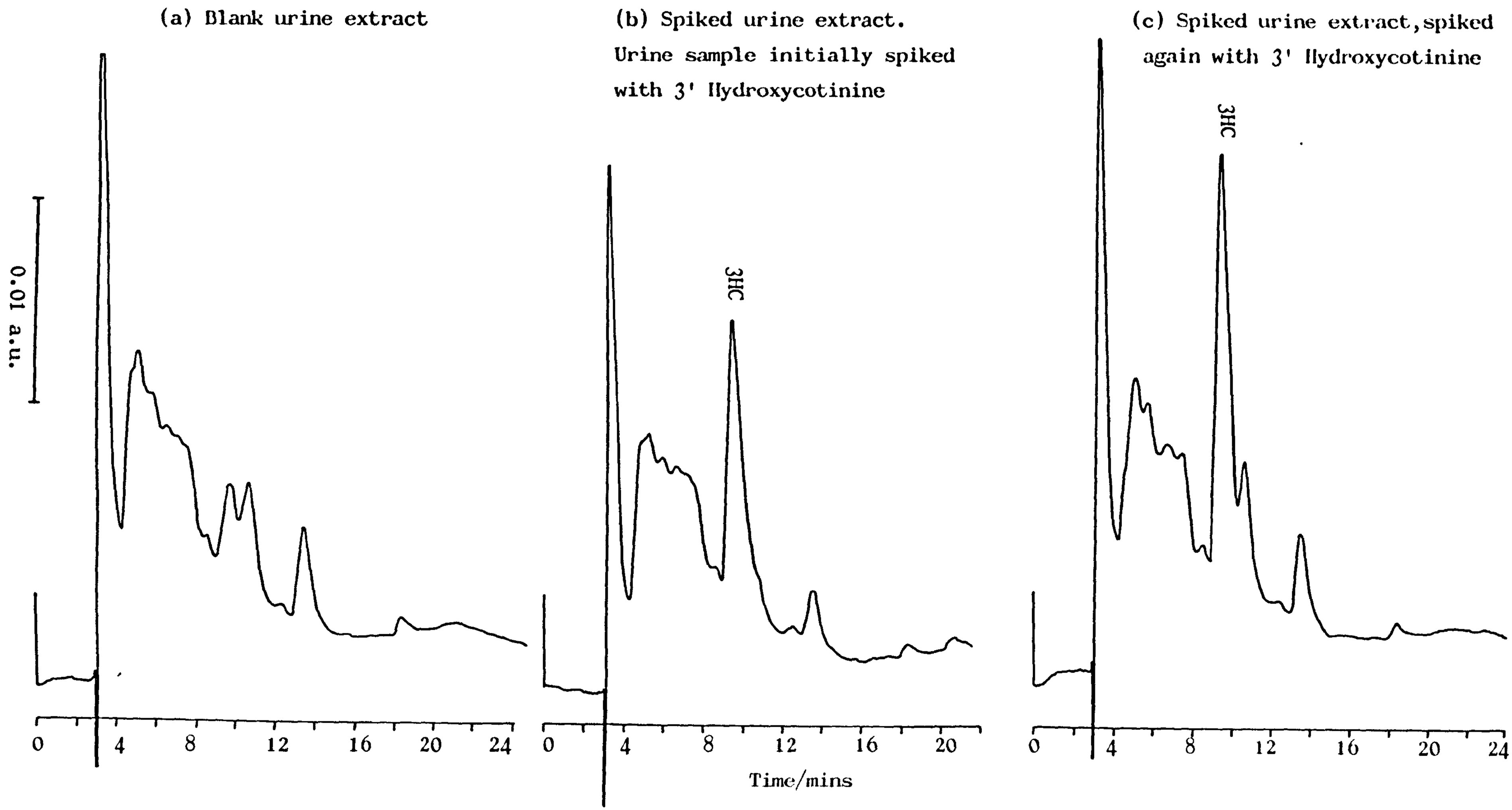


Figure 4.29: Extraction Procedure 7, applied to a urine sample, blank and spiked with 3' Hydroxycotinine (≡ 10 μg ml^{-1}), female, non-smoker. **Parameters:** see figure 4.2 except for Flow Rate: 1.2 ml min^{-1}. Gradient programme #6, 17% → 53%B over 18 mins.

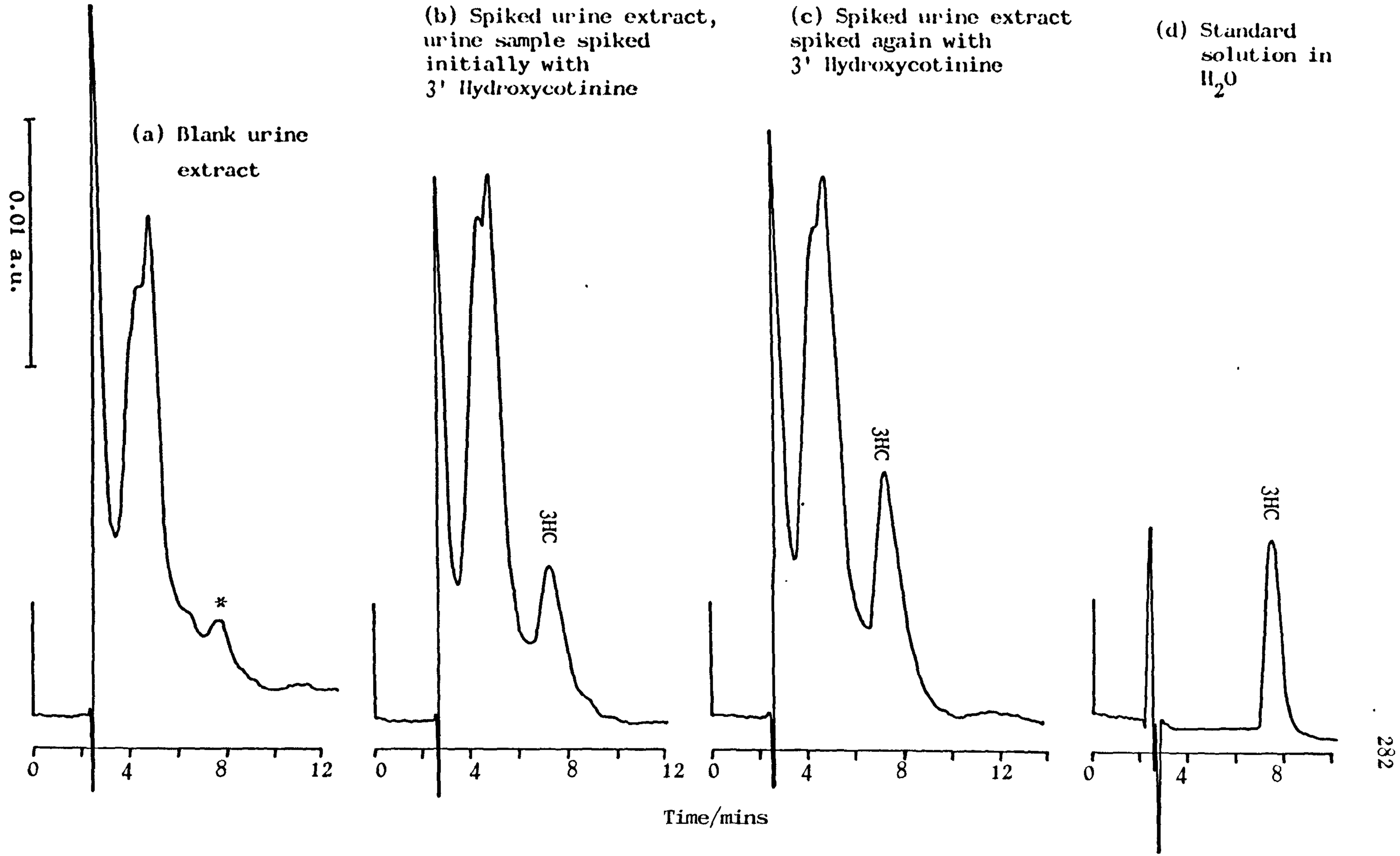


Figure 4.30: Extraction Procedure 8 (figure 4.20) applied to a urine sample.
Parameters: see figure 4.2 except for Flow Rate: 1 ml min^{-1}
Gradient programme #6, 17% → 57%B over 20 mins.

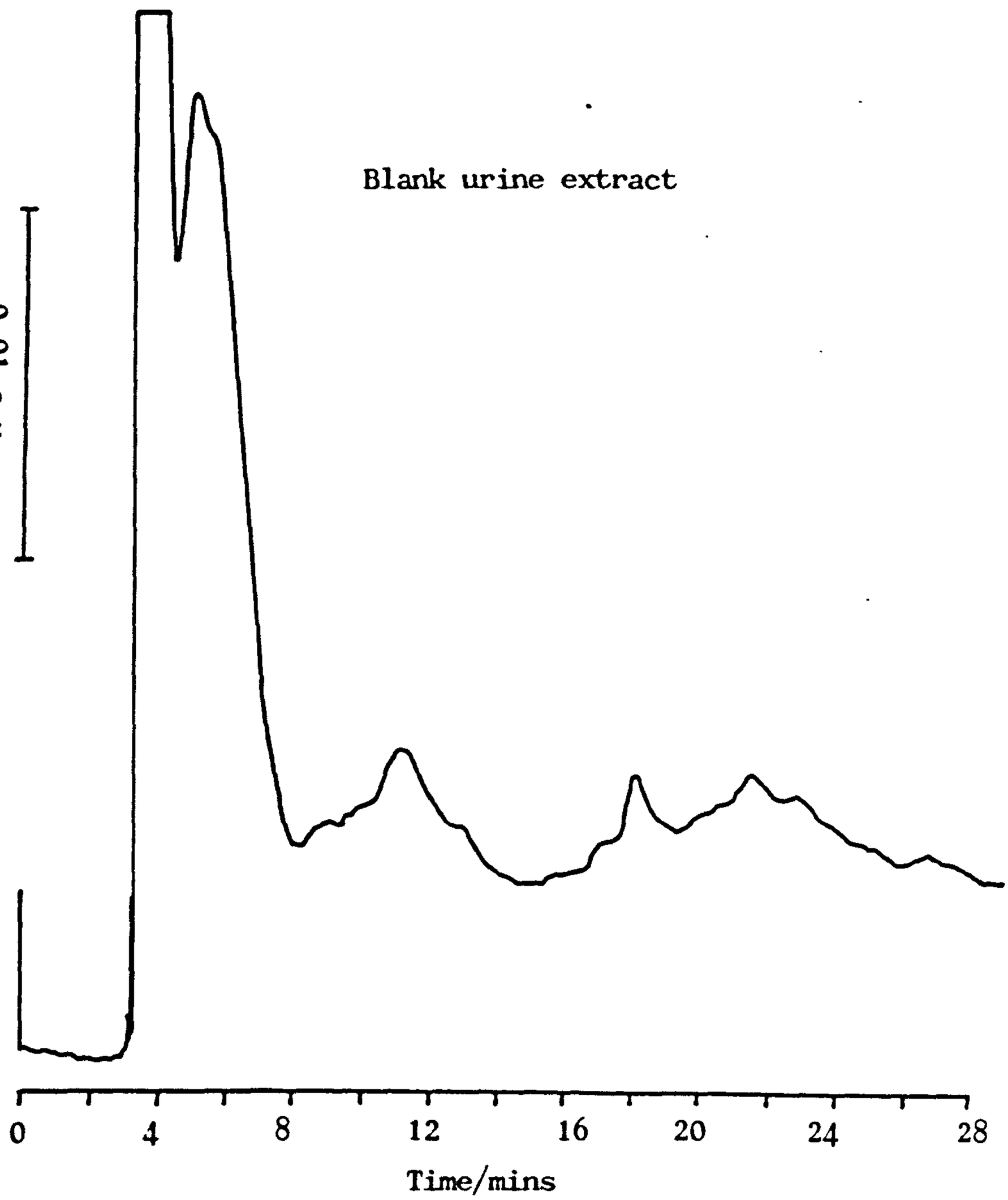
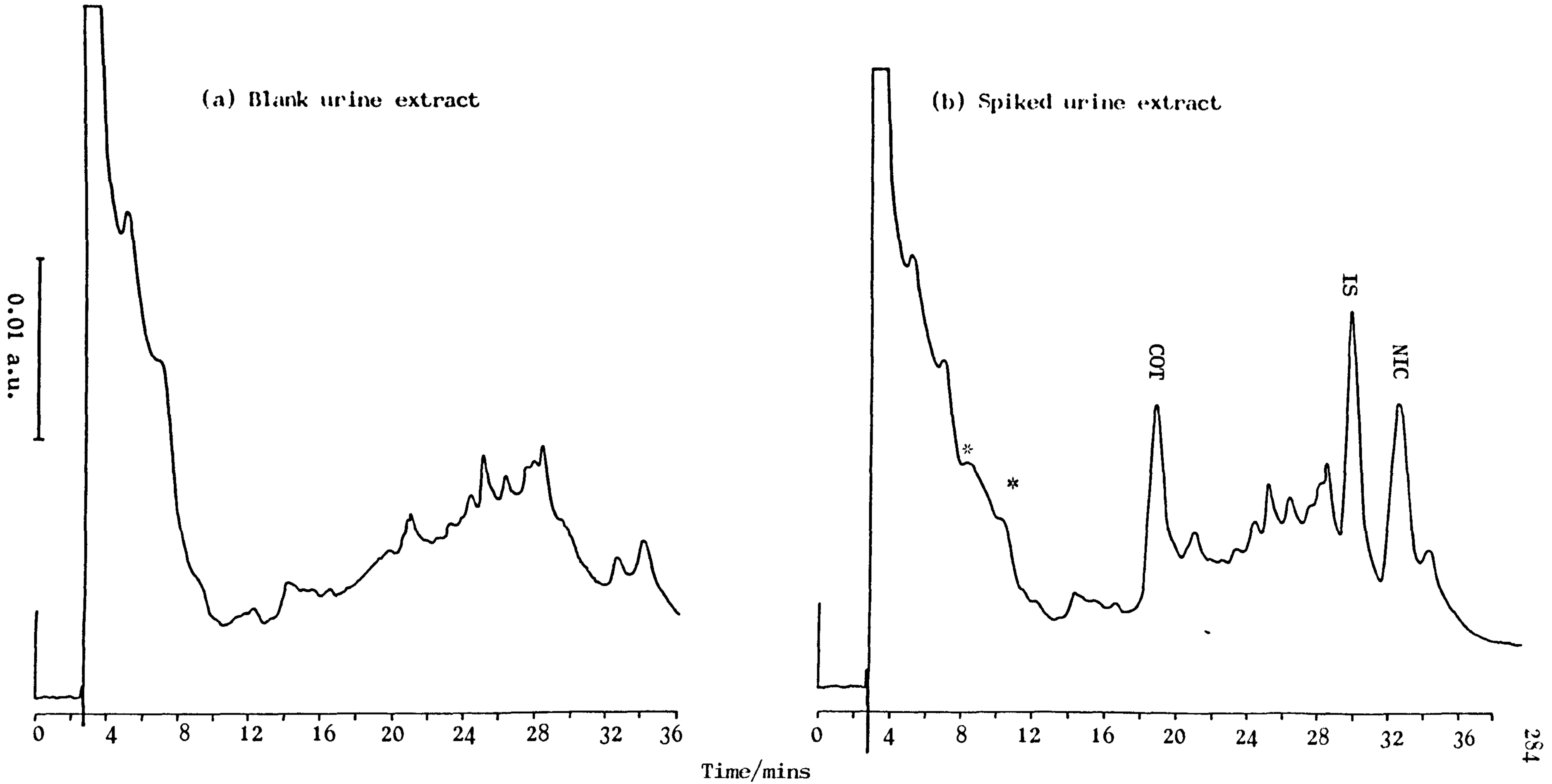


Figure 4.31: Extraction Procedure 8 (figure 4.20) applied to a urine sample, blank and spiked (≡ 10 μg ml^{-1}), (female, non-smoker). **Parameters:** see figure 4.2 except for Flow Rate: 1 ml min^{-1}. Gradient programme #6, 4 min initial hold at 17%B, 17% → 57%B over 20 mins



4.8.4 Reassessment of the extraction procedure and further investigations

The modification of the gradient programme in order to include 3' hydroxycotinine in the analysis and the problems with the clean-up procedure, discussed above, necessitated re-examination of the 'best' clean-up/extraction procedure developed, to date, and its comparison with other possible schemes. Two different clean-up procedures were compared with extraction procedure 8, figure 4.20, the same urine sample was used in each experiment, to establish whether it was possible to obtain a cleaner extract with all components of analytical interest extracted with satisfactory recovery rates and without interferences.

Extraction procedure 9, figure 4.32, did not include the IPA dodecylsodium sulphate. The sample was adjusted to pH 2.0 and passed through the Sep Pak. At pH 2.0 the components of interest were not expected to be retained by the C_{18} packing material in the Sep Pak. Having collected the effluent from the Sep Pak, the pH was adjusted to 7.0 and the scheme was completed with the steps from extraction procedure 1, figure 4.1. Extraction procedure 9 was applied to a standard mixture in water, blank and spiked urine samples, and the resulting chromatograms are presented in figures 4.33 and 4.34. The recovery values are reported in table 4.1. N' acetyl nornicotine was included in this experiment. The reason it had been discarded was due to unsatisfactory extraction by scheme 6, figure 4.12.

Figure 4.32: Extraction Procedure 9

Sample (20 ml) : urine or standard mixture in H_2O (10 μg ml^{-1})

↓

Add IS as a 200 μl spike of a 1000 μg ml^{-1} solution (≡ 10 μg ml^{-1})

↓

Adjust pH to 2.0 - 2.1 with conc. HCl

↓

Pass sample (10 ml) through a Sep Pak and collect

↓

Adjust pH to 7.0 with NaOH

↓

Pass through another Sep Pak and discard effluent

↓

Wash Sep Pak with H_2O (5 ml) and discard

↓

Elute components of interest from Sep Pak with 100% methanol (5 ml)

↓

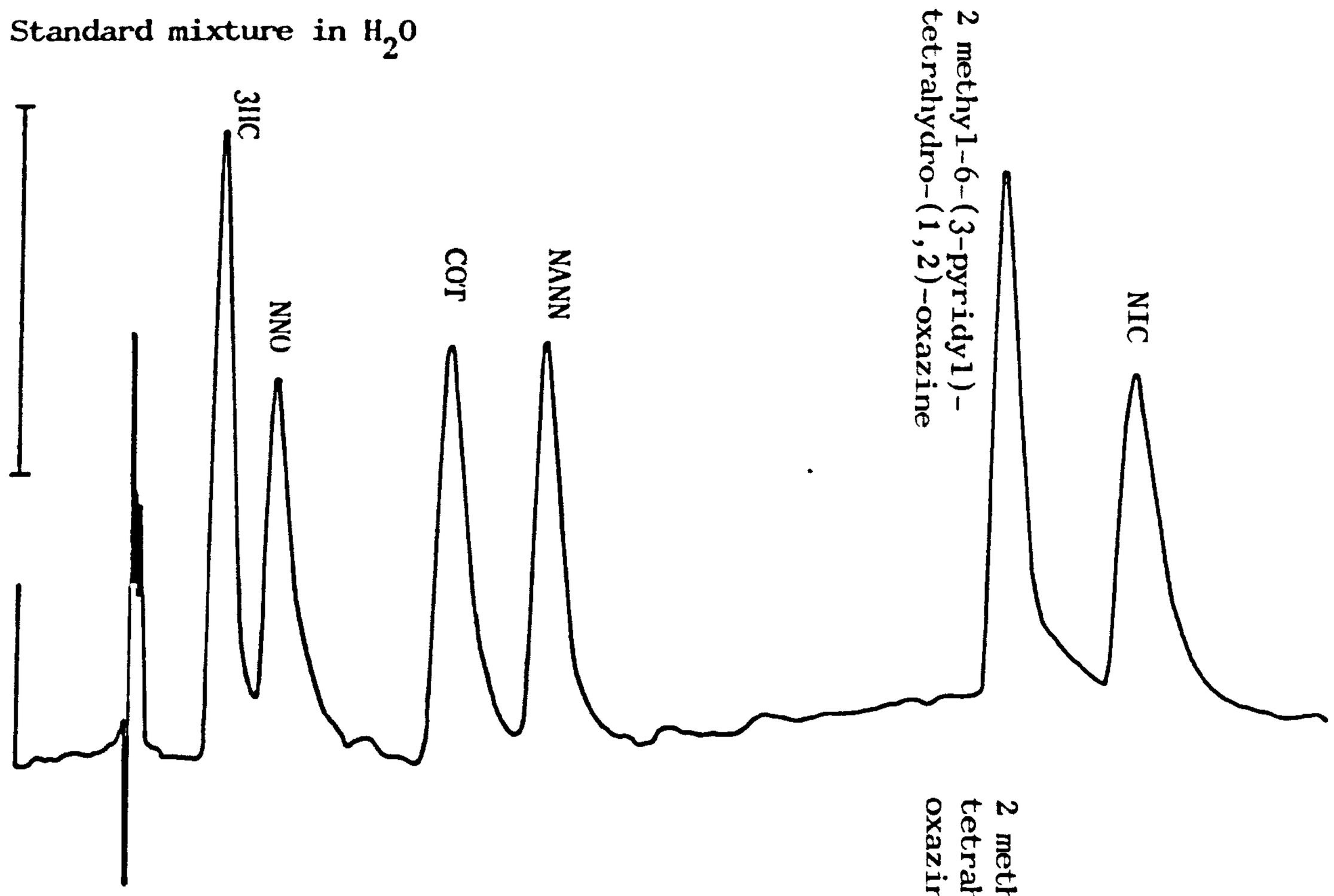
Make extract volume 10 ml by adding 0.2% H_3PO_4, pH 7.25

↓

Inject

Figure 4.33: Extraction Procedure 9 (figure 4.42) applied to a standard mixture (≡ 10 μg ml^{-1}) in H_2O.
Parameters: see figure 4.2 except for Flow Rate: 1 ml min^{-1}
Gradient programme #6, 4 min initial hold at 25%B, 25% → 50%B over 20 mins

(a) Standard mixture in H_2O



(b) Extract of standard mixture

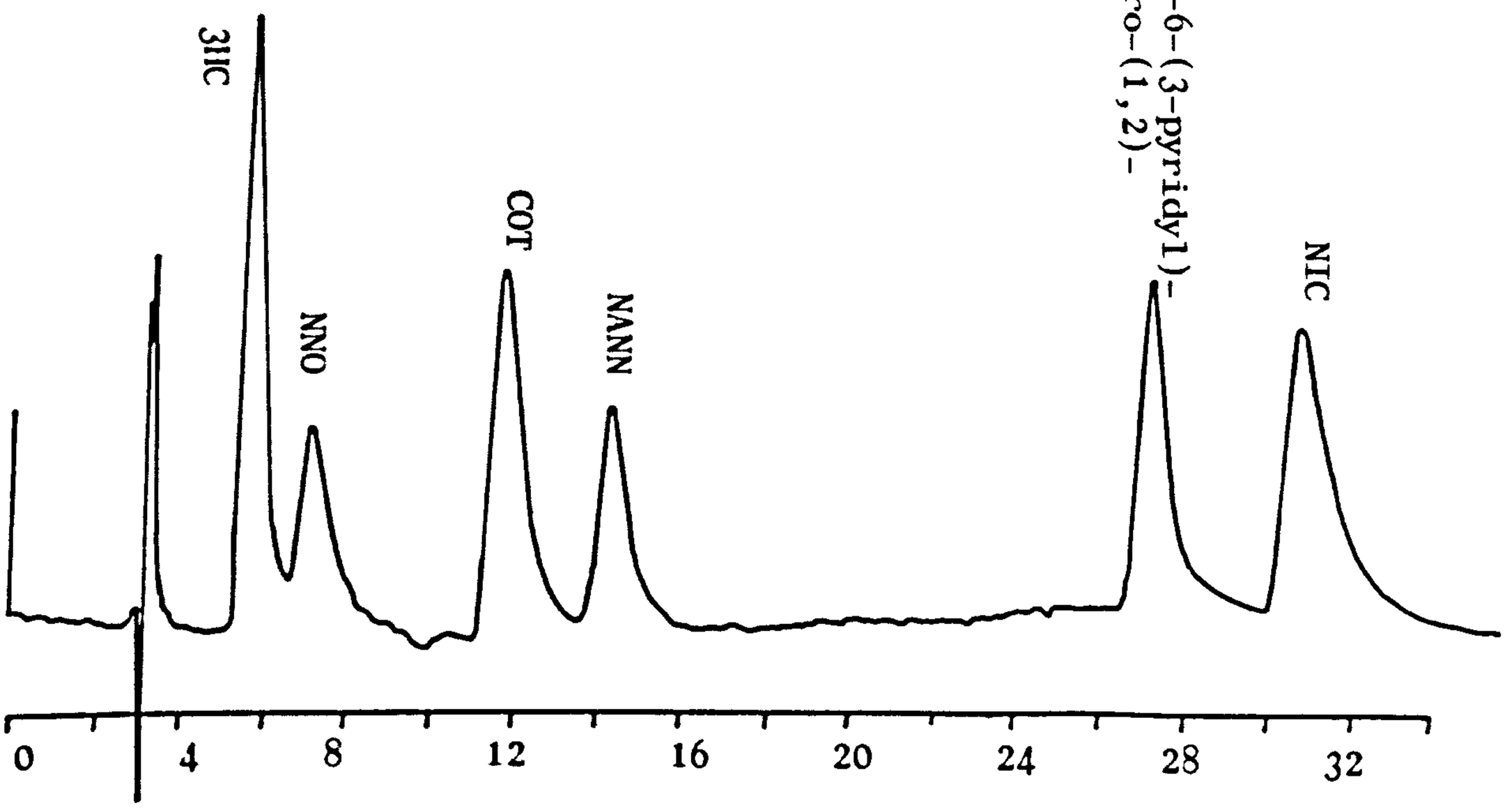
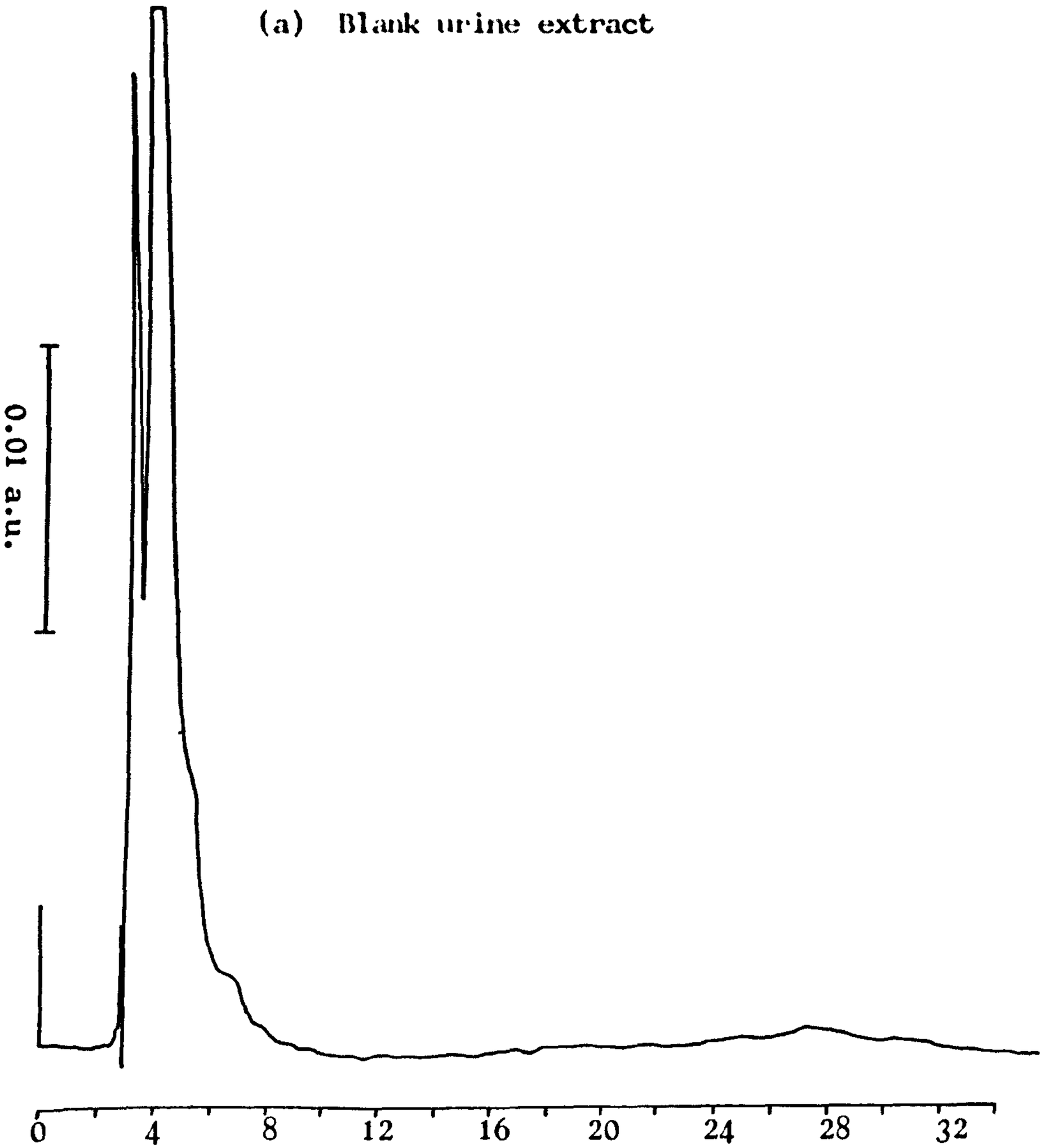


Figure 4.34: Extraction Procedure 9 (figure 4.32) applied to a urine sample, blank and spiked (≡ 10 μg ml^{-1}), female, non-smoker. **Parameters:** see figure 4.33



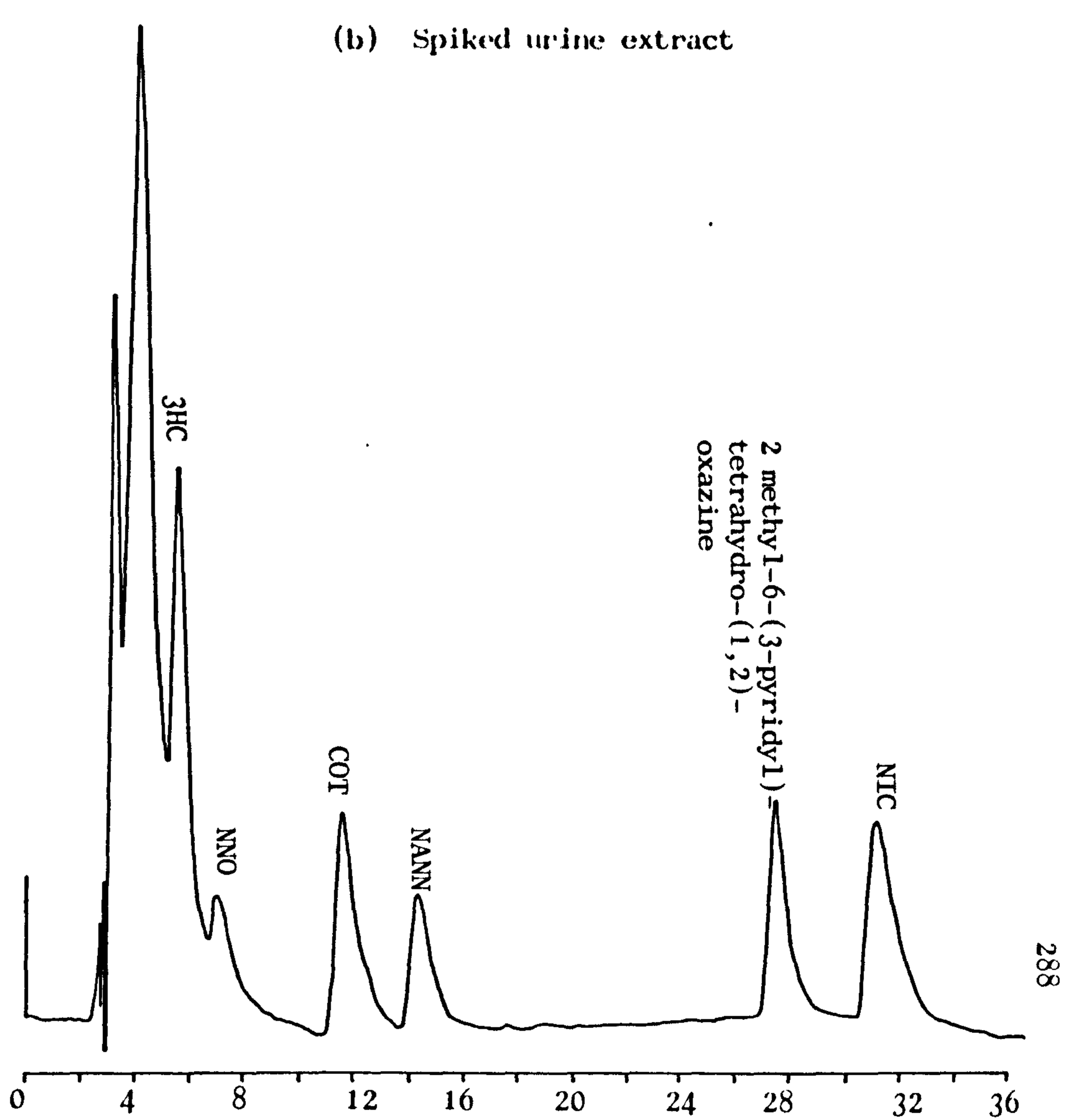


TABLE 4.1: Extraction yields obtained using extraction procedure 9

	Extraction yield from a standard solution in H_2O (%)	Extraction yield from a spiked urine sample (≡ 10 μg ml^{-1}) (%)
3' Hydroxycotinine	84	79*
Nicotine-1'-N-oxide	43	26*
Cotinine	77	71
N' Acetyl nornicotine	48	45
2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine	55	55
Nicotine	79	83

* on the tail of another peak

The recovery values obtained using extraction procedure 9, figure 4.32, were not very promising and although the clean-up of the urine sample was impressive, on extracting other urine samples, it was again noted that another component, which interfered with the 3' hydroxycotinine/nicotine-1'-N-oxide peaks, was co-extracted, as shown in figure 4.35.

When this extraction procedure was applied to a caffeine standard, caffeine was lost during the first step as it was retained by the Sep Pak.

In all previous experiments involving the use of an IPA, dodecylsodium sulphate, 8 mg for every 4 ml of sample, had been used. Substitution of this IPA with one having a C_7 chain resulted in the loss of all components when extraction procedure 8, figure 4.20, was applied.

Figure 4.35: Extraction Procedure 9 (figure 4.32) applied to a urine sample, blank and spiked with 3' llydroxycotinine (≡ 10 μg ml^{-1}), female, non-smoker. Parameters: see figure 4.33.

(a) Blank urine extract

(b) Spiked urine extract

3HC

(c) Blank urine extract, spiked after extraction with 3' llydroxy-cotinine

3HC

0.01 a.u.

0 4 8 12 16 Time/mins

In another experiment using one tenth of the IPA previously used, i.e. 0.8 mg for every 4 ml of sample, revealed that the recovery of cotinine and 3' hydroxycotinine in particular was unsatisfactory, as shown in figure 4.36. Therefore, the use of dodecylsodium sulphate, 8 mg for every 4 ml of sample, was continued.

For comparison with extraction procedure 9, figure 4.32, extraction procedure 8, figure 4.20, was re-examined using the same blank urine sample. Chromatograms showing extracts of a standard mixture, blank and spiked urine samples, are presented in figures 4.37 and 4.38. The recovery values are reported in table 4.2.

TABLE 4.2: Extraction yields obtained using extraction procedure 8

	Extraction yield from a standard solution in H_2O (%)	Extraction yield from a spiked urine sample ($\equiv$ 10 $\mu g\ ml^{-1}$) (%)
3' Hydroxycotinine	78	66*
Nicotine-1'-N-oxide	75	75*
Cotinine	82	83
2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine	82	83
Nicotine	89	100

* on the tail of another peak

One other extraction procedure was investigated, the detail is presented in figure 4.39, extraction procedure 10. As in extraction procedure 9, figure 4.32, the sample, pH adjusted to 2.0, was passed through the Sep Pak and the effluent collected.

Figure 4.36: Extraction Procedure 6 (figure 4.12) (using 4 mg IPA for a 20 ml sample), applied to a standard mixture (≡ 10 μg ml^{-1}) in H_2O. **Parameters:** see figure 4.2 except for Flow Rate: 1 ml min^{-1} and Gradient programme #6, 4 min initial hold at 25%B, 25% → 55%B over 20 mins

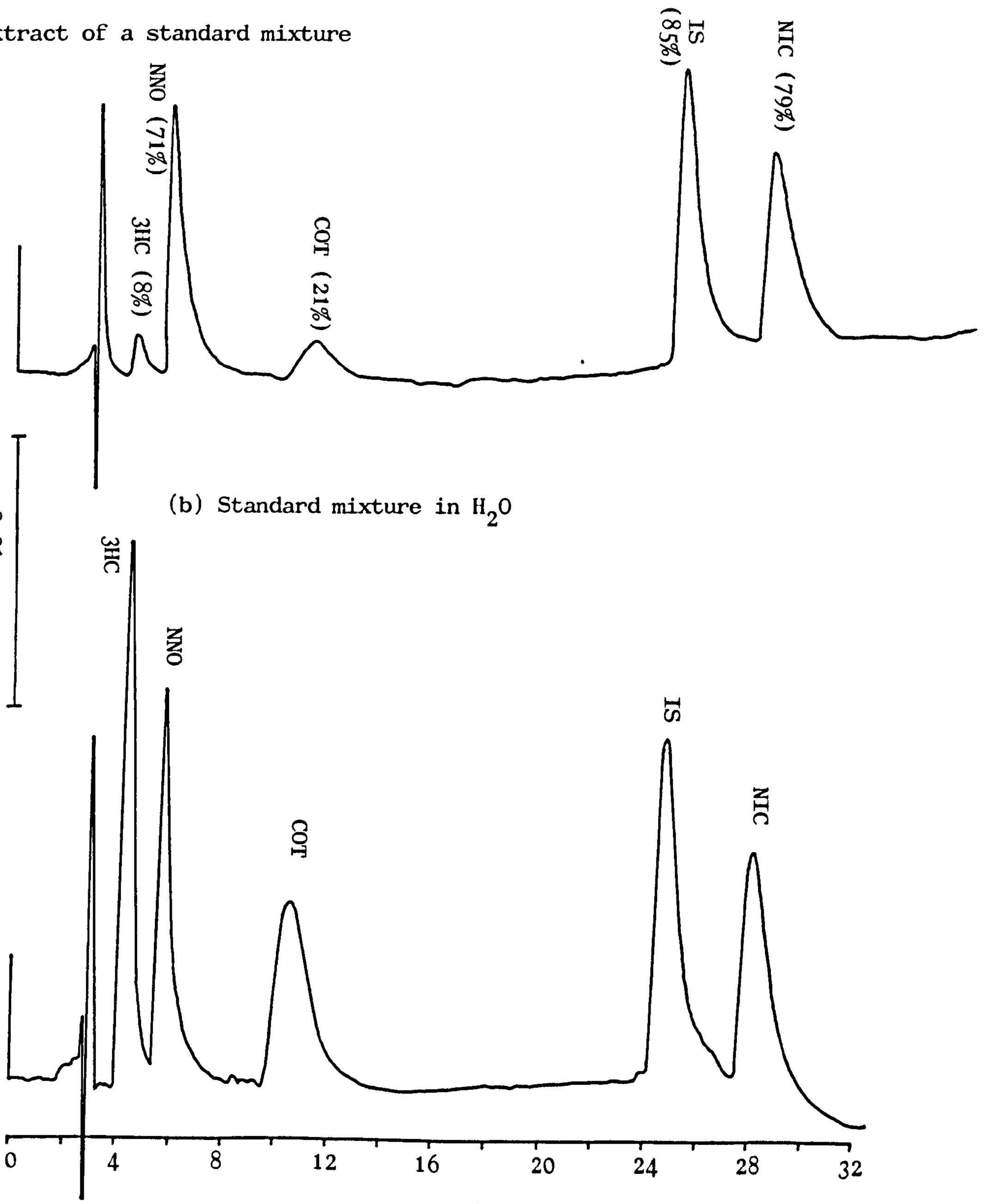


Figure 4.37: Extraction Procedure 8 (figure 4.20), applied to a standard mixture (≡ 10 μg ml^{-1}) in H_2O
Parameters: see figure 4.36

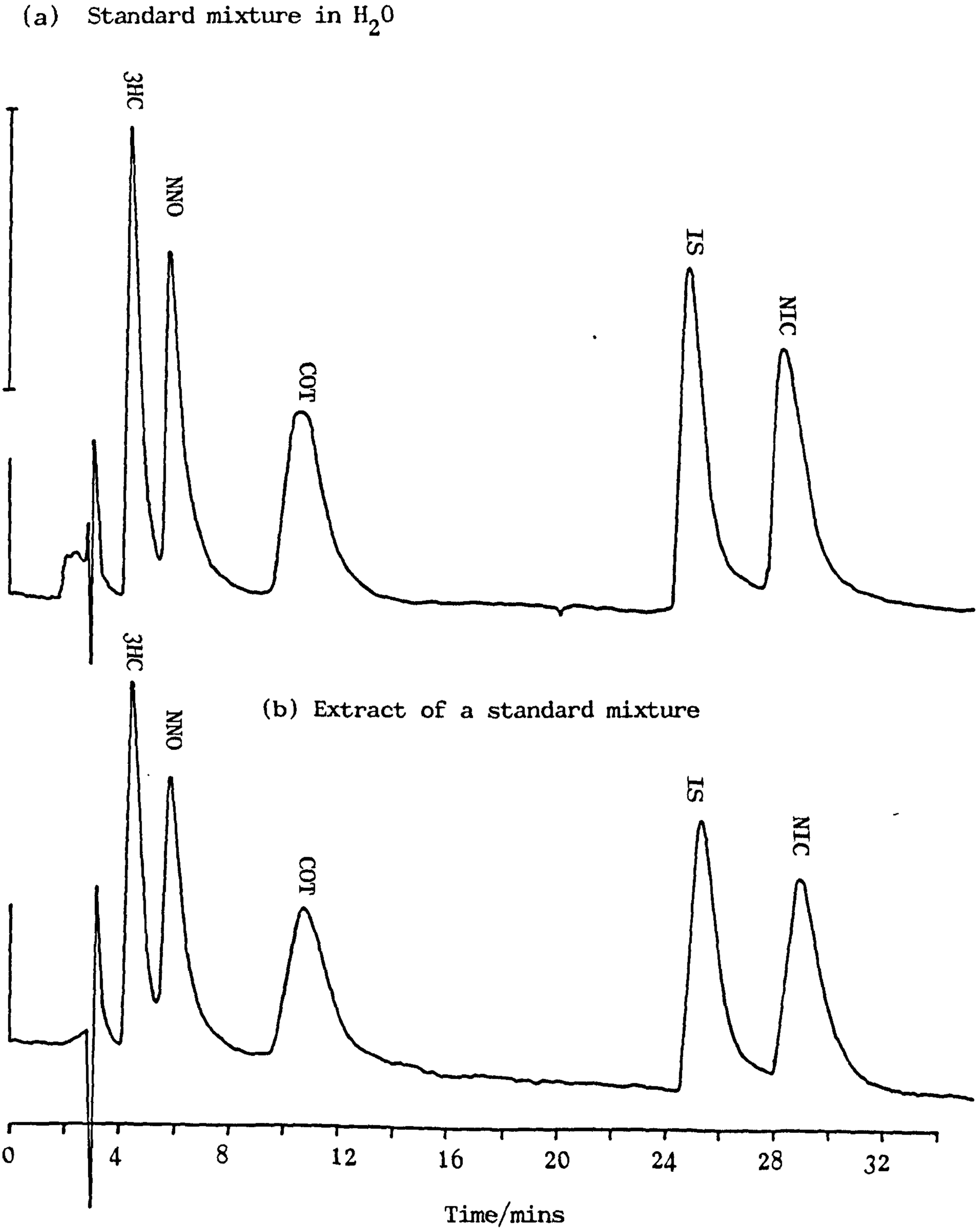


Figure 4.38: Extraction Procedure 8, figure 4.20, applied to a urine sample, blank and spiked (≡ 10 μg ml^{-1}), female, non-smoker. **Parameters:** see figure 4.36.

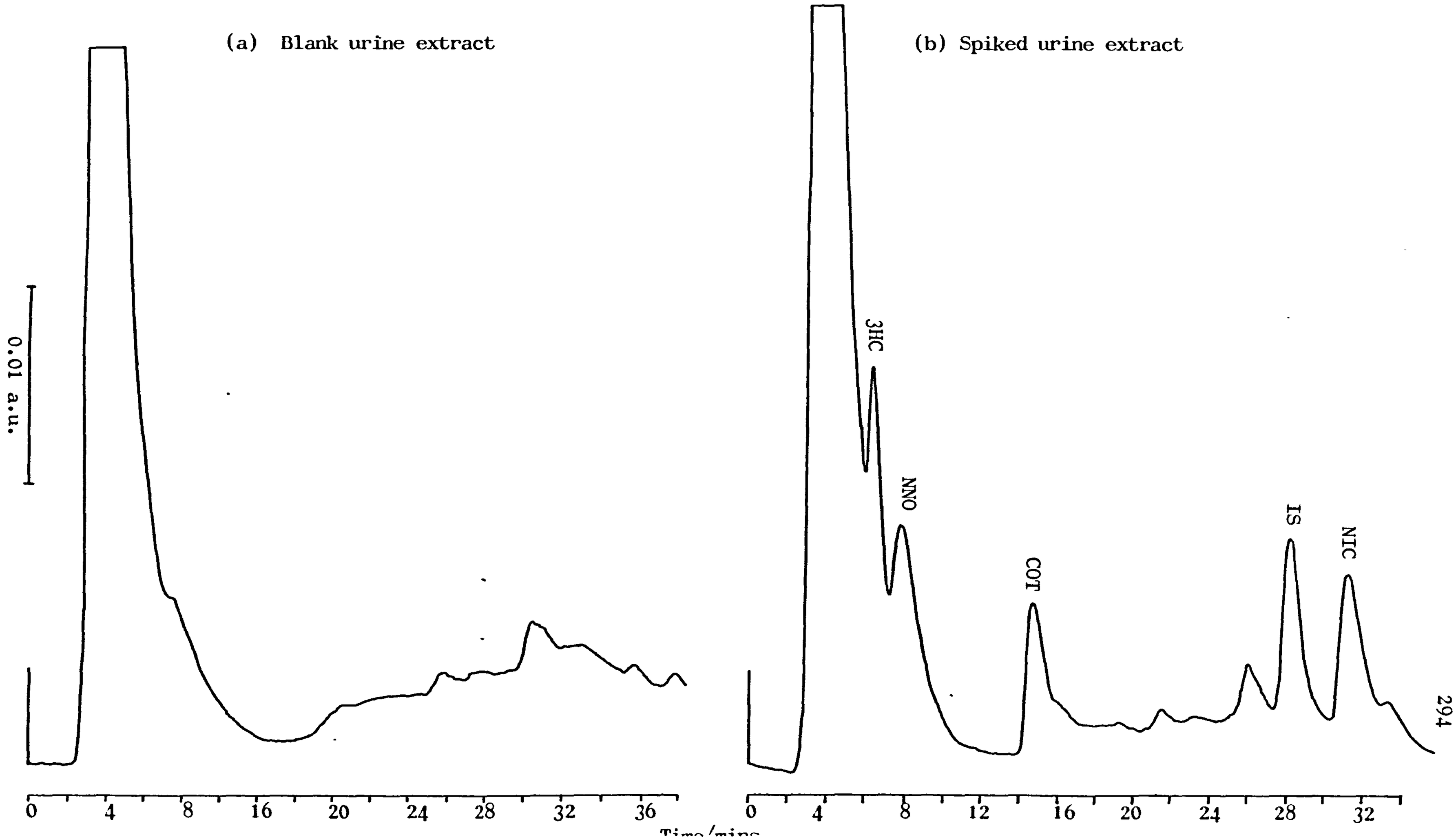
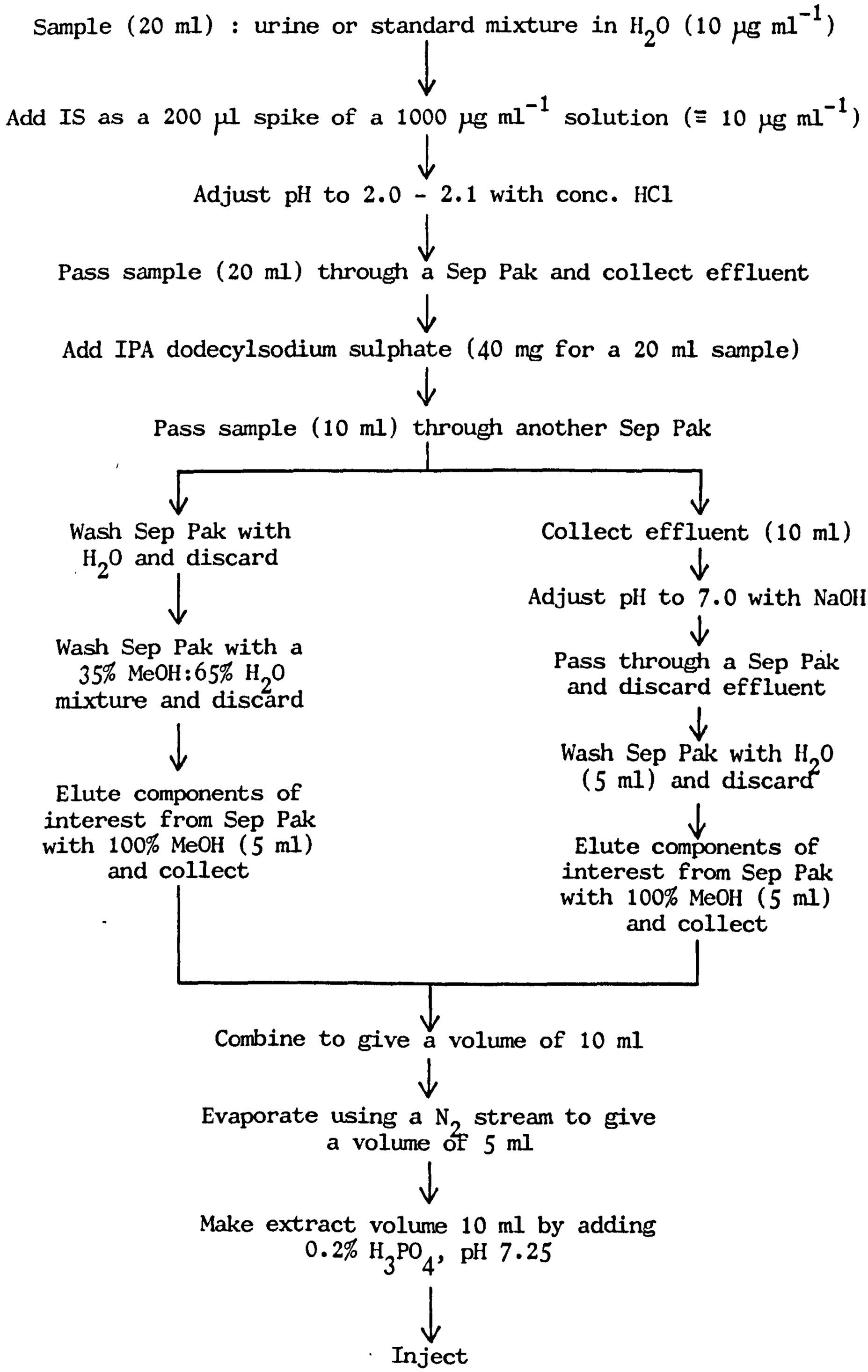


Figure 4.39: Extraction Procedure 10



The remaining steps in the scheme were the same as those followed in procedure 8, figure 4.20. The chromatograms, showing the extracts obtained when extraction procedure 10 was applied to a standard mixture in water, blank and spiked urine samples, are presented in figures 4.40 and 4.41 and the recovery values are reported in table 4.3.

TABLE 4.3: Extraction yields obtained using extraction procedure 10

	Extraction yield from a standard solution in H_2O (%)	Extraction yield from a spiked urine sample ($\equiv$ 10 $\mu g\ ml^{-1}$) (%)
3' Hydroxycotinine	95	60 *
Nicotine-1'-N-oxide	94	29 *
Cotinine	98	112
2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine	78	43
Nicotine	90	74

* on the tail of another peak

As far as extractions of the components of interest from urine were concerned, extraction procedure 8, figure 4.20, was superior with respect to the recovery values observed, an important factor when the concentrations of the components of interest are low. Extraction procedure 10 was abandoned, although the extraction yields of the components of interest from a standard mixture in water were excellent, those obtained when a spike urine sample was examined were disappointing and the clean-up of the urine sample did not show any improvement over that achieved by either extraction

Figure 4.40: Extraction Procedure 10, figure 4.39, applied to a standard mixture (≡ 10 μg ml^{-1}) in H_2O
Parameters: see figure 4.36

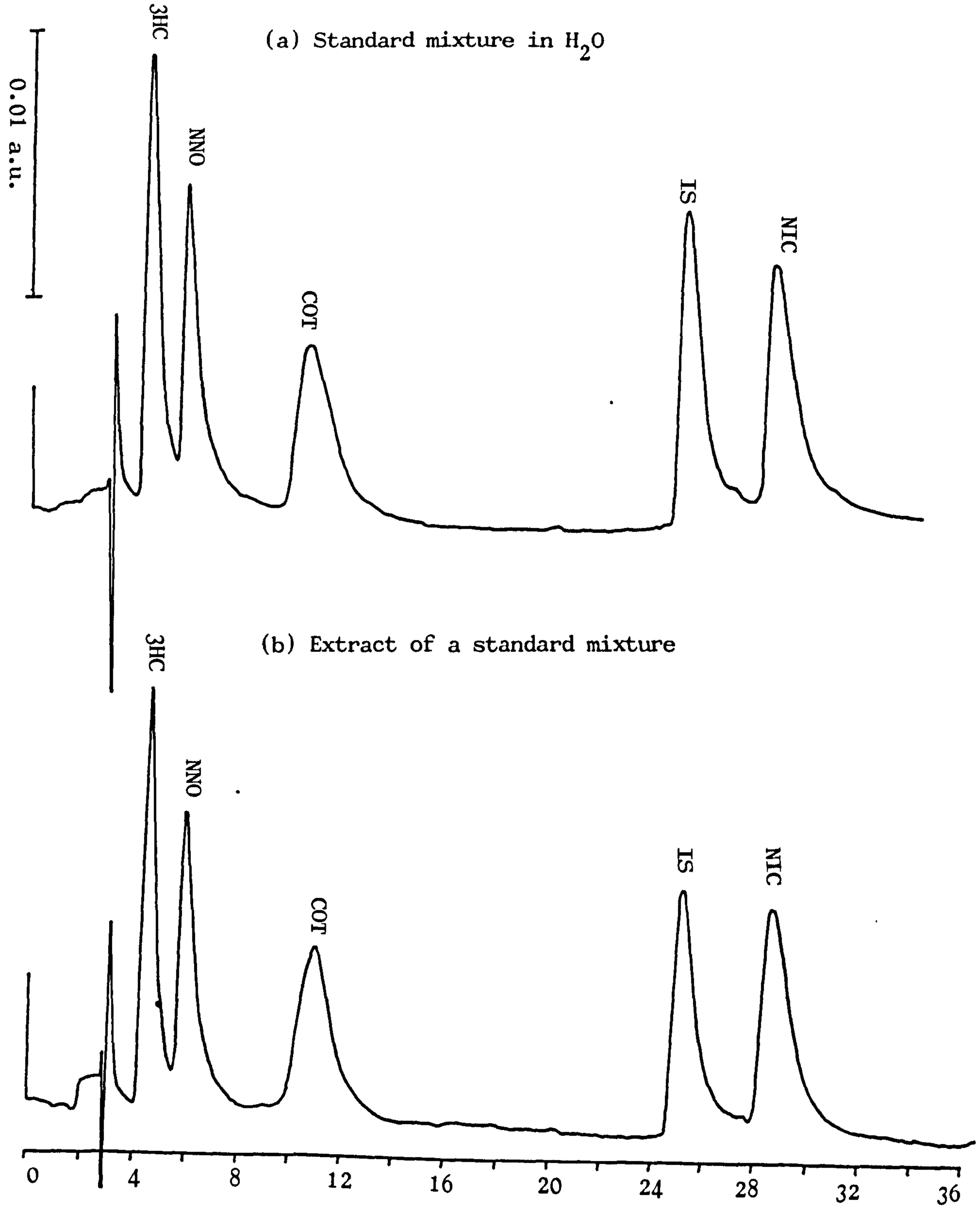
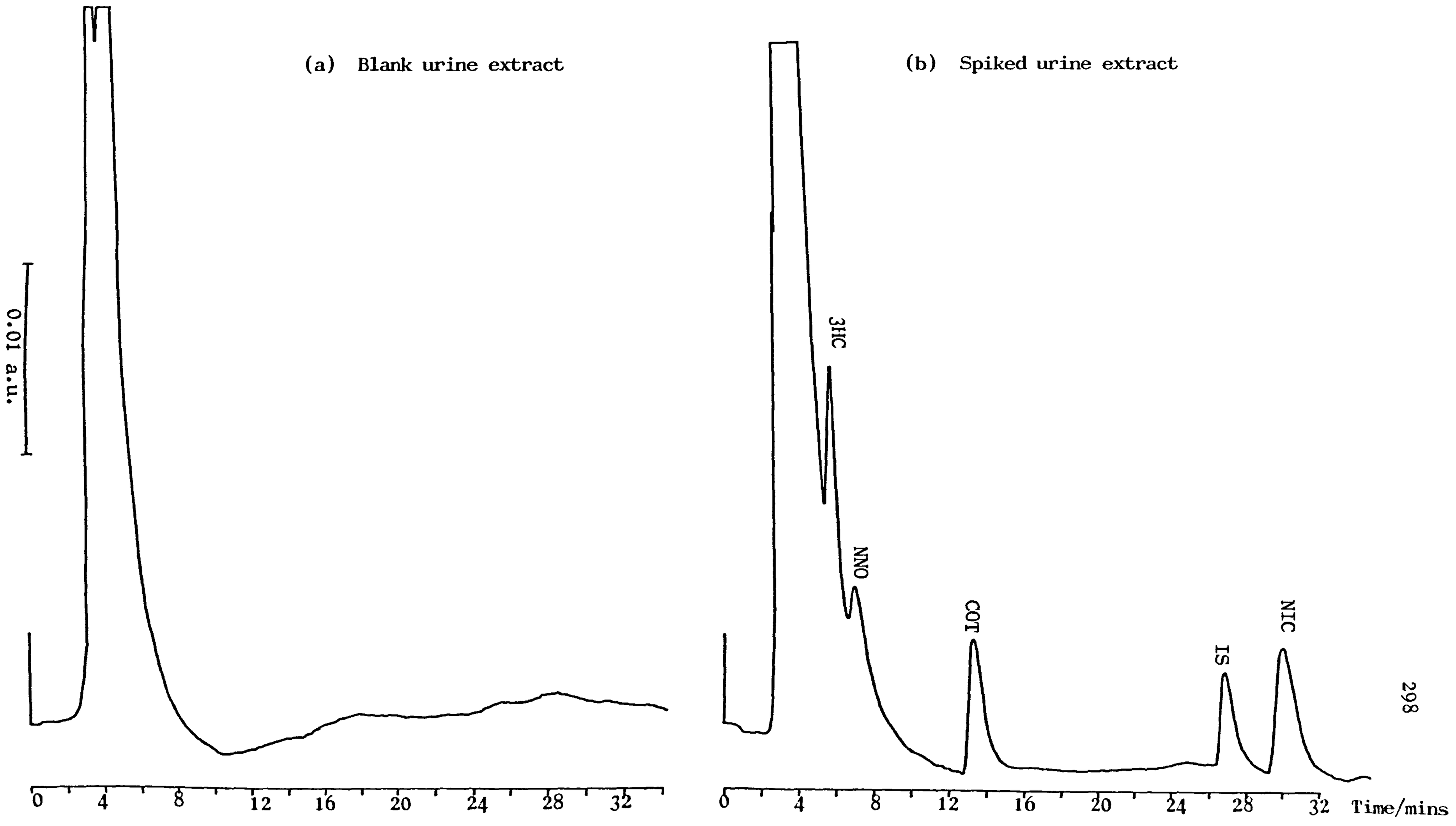


Figure 4.41: Extraction Procedure 10, figure 4.39, applied to a urine sample, blank and spiked (≡ 10 μg ml^{-1}), female, non-smoker. **Parameters:** see figure 4.36.



procedures 8 or 9. Both extraction procedures 8 and 9 (figures 4.20 and 4.32) have shown the co-extraction of a component which interferes with 3' hydroxycotinine, or possibly nicotine-1'-N-oxide; however, in the absence of any other possible clean-up/extraction procedures, procedures 8 and 9 were applied to smokers' urine samples.

A blank urine sample together with the same urine sample spiked with nicotine, cotinine, 3' hydroxycotinine and nicotine-1'-N-oxide ($\equiv$ 7 $\mu g\ ml^{-1}$, $\equiv$ 5 $\mu g\ ml^{-1}$ and $\equiv$ 3 $\mu g\ ml^{-1}$), the internal standard being added in all cases ($\equiv$ 10 $\mu g\ ml^{-1}$), were extracted using scheme 8, figure 4.20. Chromatograms of the extracts are shown in figure 4.42, chromatograms of the standard solutions being included for comparison. The sample clean-up was not satisfactory on this occasion, and so it was not possible to identify nicotine-1'-N-oxide or 3' hydroxycotinine. As the concentrations of the components of interest were decreased, it was increasingly difficult to identify any of the components of interest. Unfortunately poor column resolution contributed to the difficulty in this experiment. The need for an excellent clean-up procedure, good column efficiency and the highest possible extraction yields was apparent from figure 4.42. Concentration of the extract is also essential and peak identification should be confirmed, as it has been, by spiking the extract with the components of interest.

Figure 4.42: Extraction Procedure 8, figure 4.20, applied to standard mixtures, blank and spiked urine samples (concentrations as specified below).
Parameters: see figure 4.36

(a) Blank urine extract

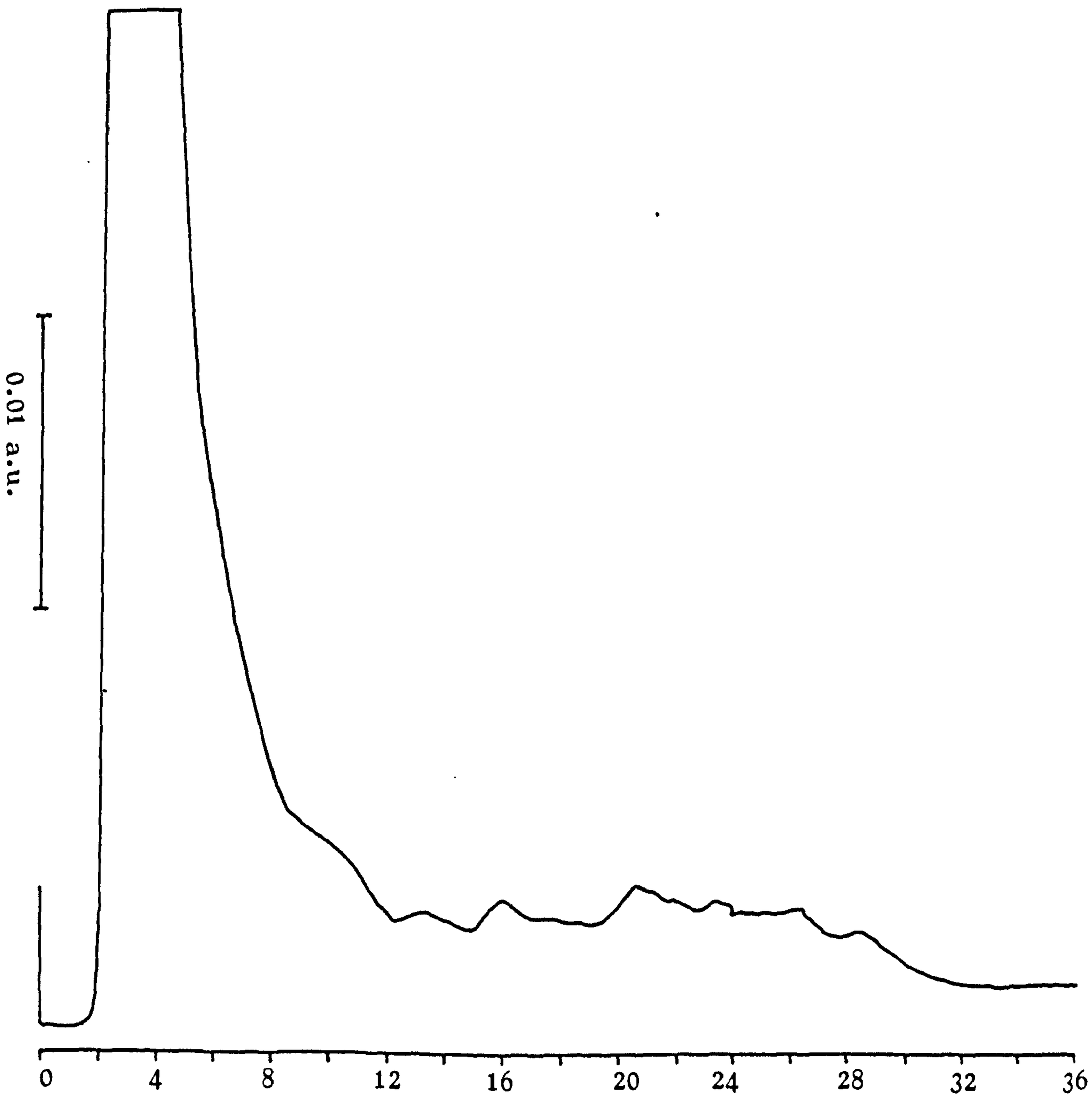
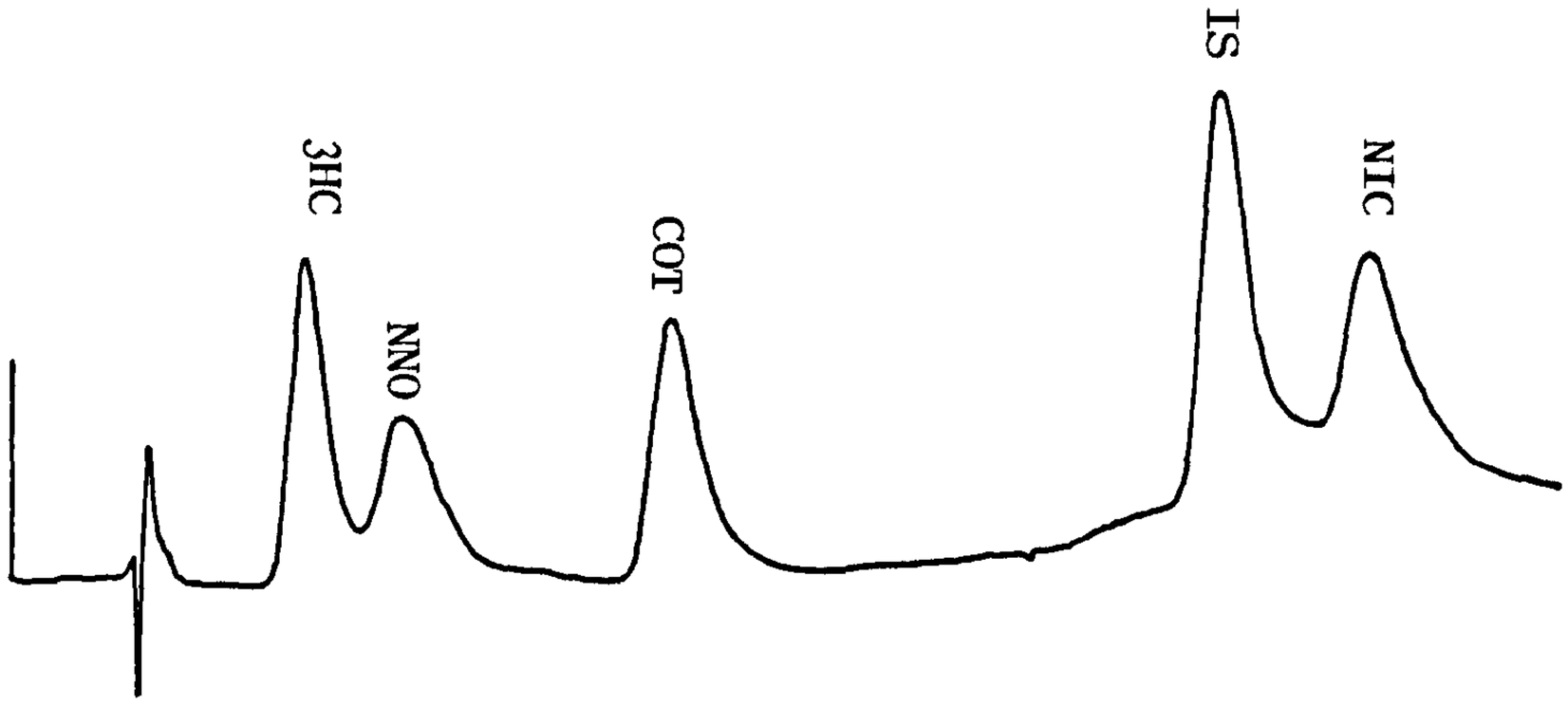


Figure 4.42 continued

(b) Extract of a 7 μg ml^{-1} standard mixture in H_2O, including IS (≡ 10μg ml^{-1})



(c) Extract of a spiked urine sample (≡ 7 μg ml^{-1}) and IS (≡ 10μg ml^{-1})

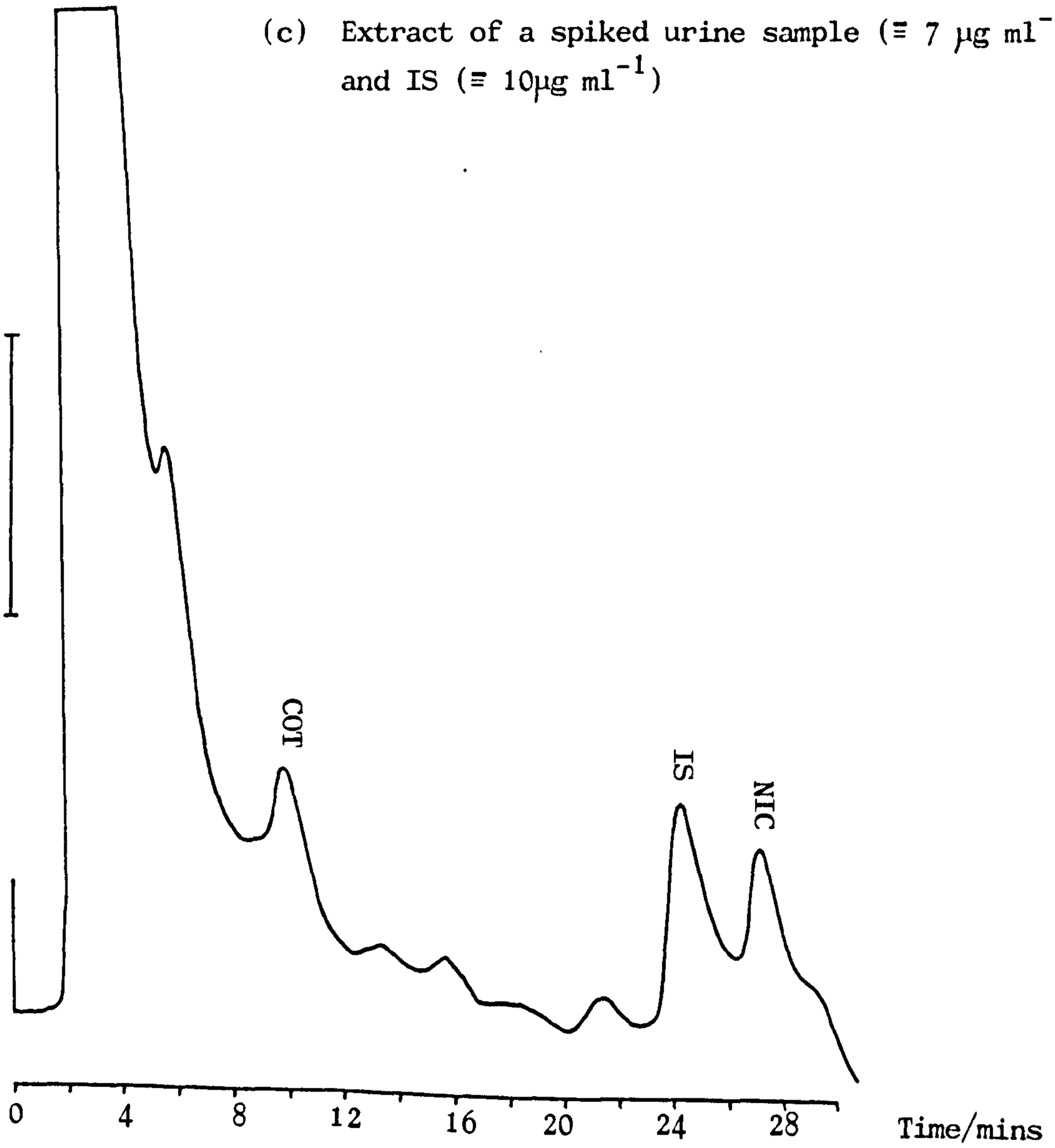
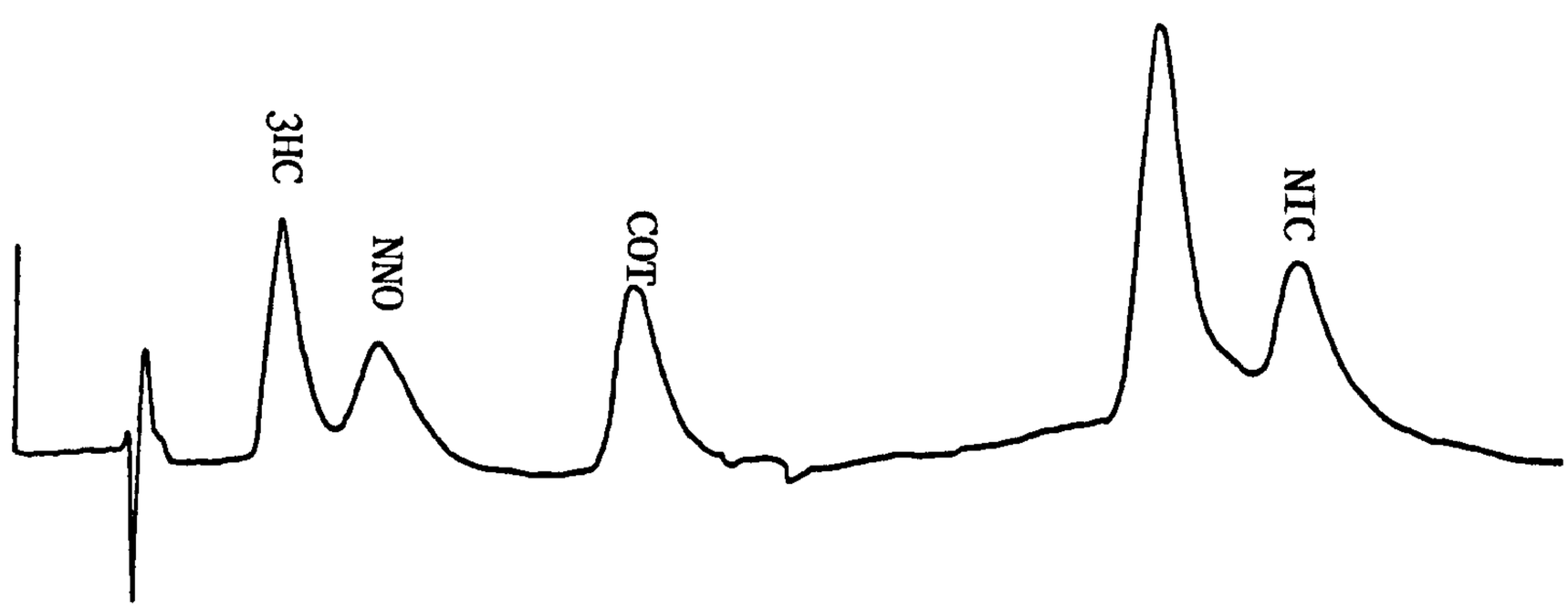


Figure 4.42 continued

(d) Extract of a 5 μg ml^{-1} standard mixture in H_2O including IS (≡ 10ug ml^{-1})



(e) Extract of a spiked urine sample (≡ 5 μg ml^{-1}) and IS (≡ 10μg ml^{-1})

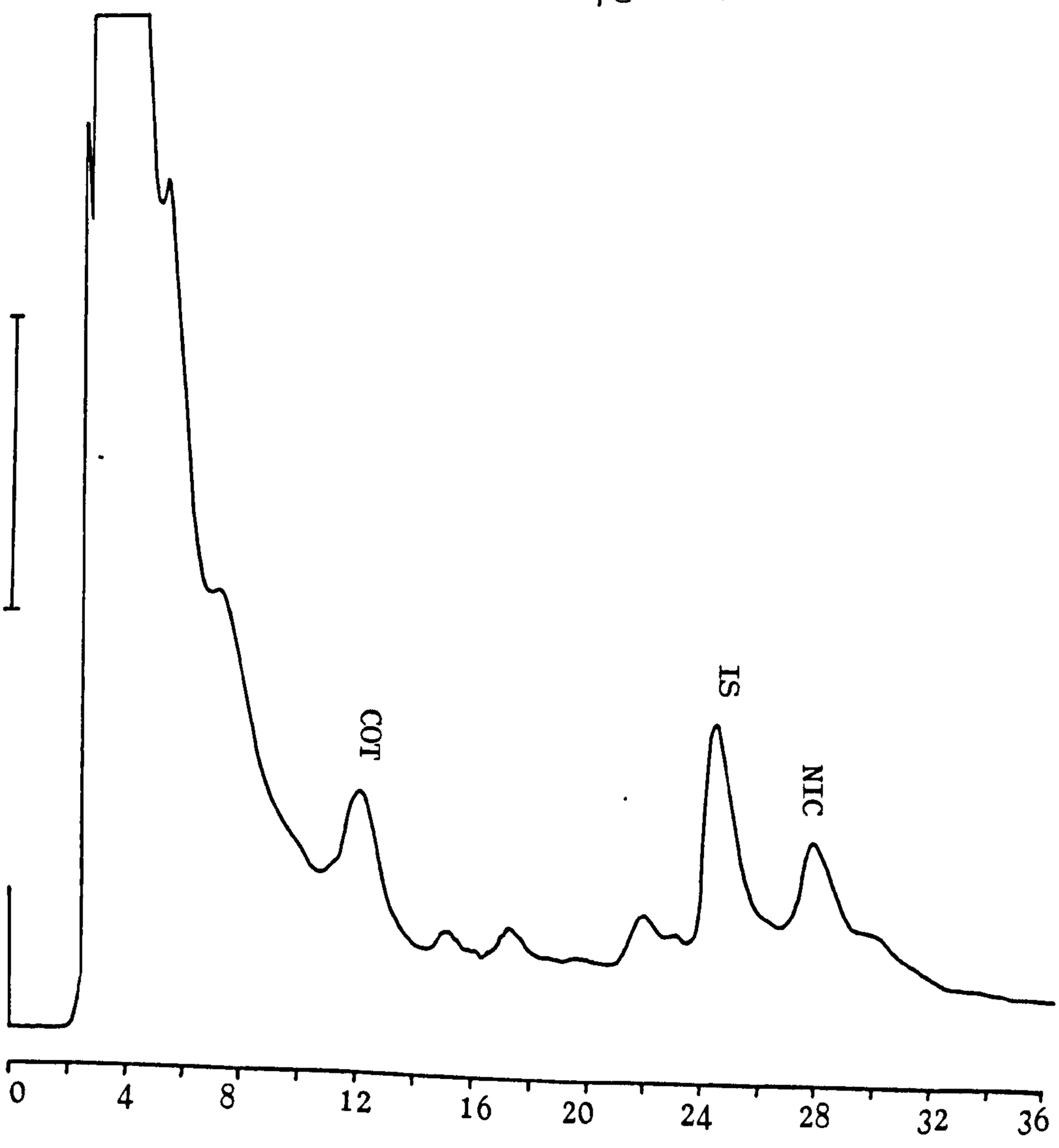
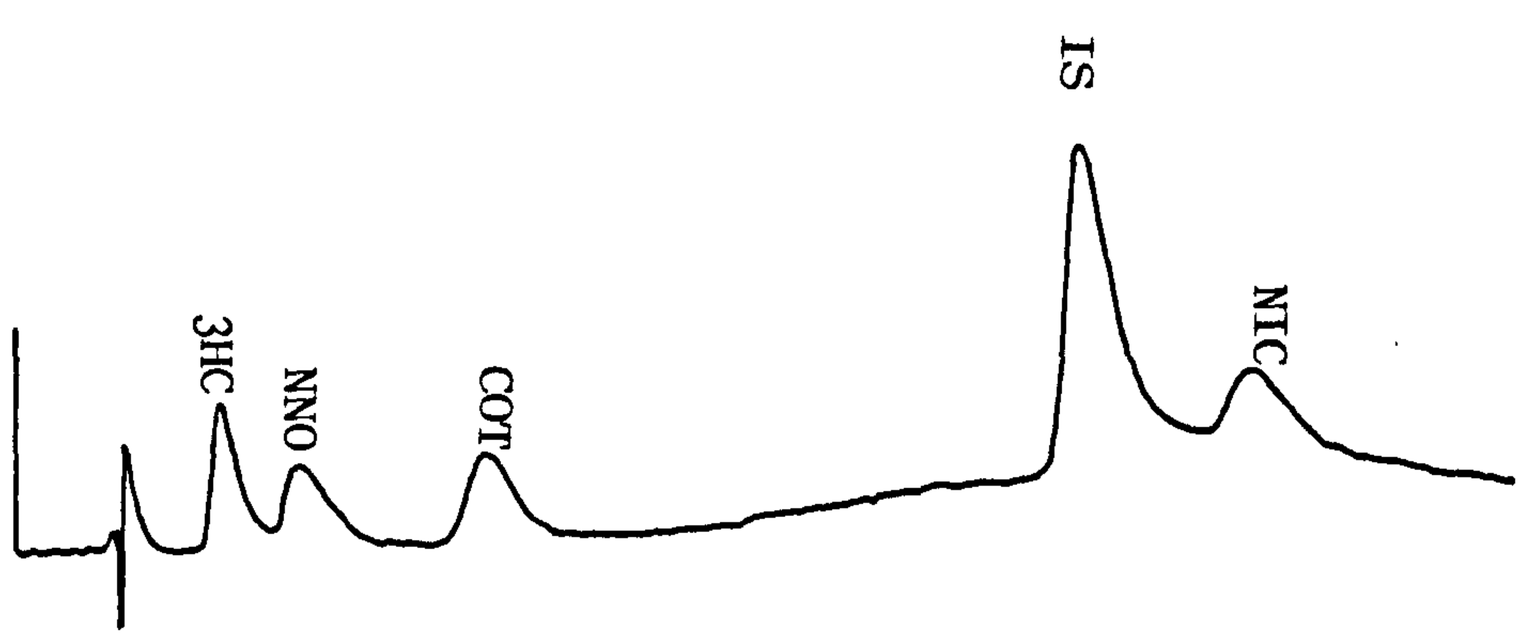
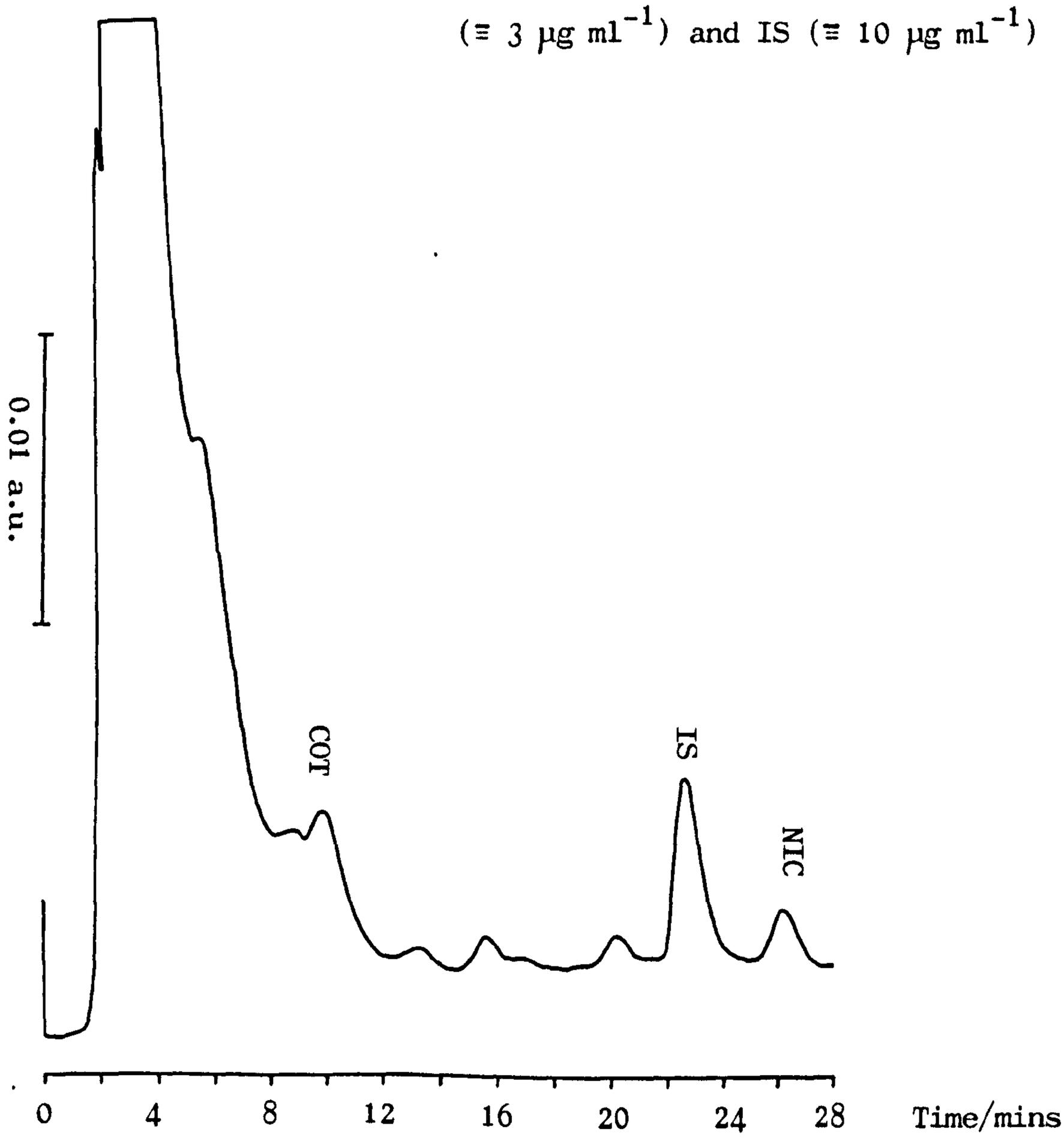


Figure 4.42 continued

(f) Extract of a 3 μg ml^{-1} standard mixture in H_2O, including IS (≡ 10 μg ml^{-1})



(g) Extract of a spiked urine sample (≡ 3 μg ml^{-1}) and IS (≡ 10 μg ml^{-1})



4.8.5 Concentration of the extract

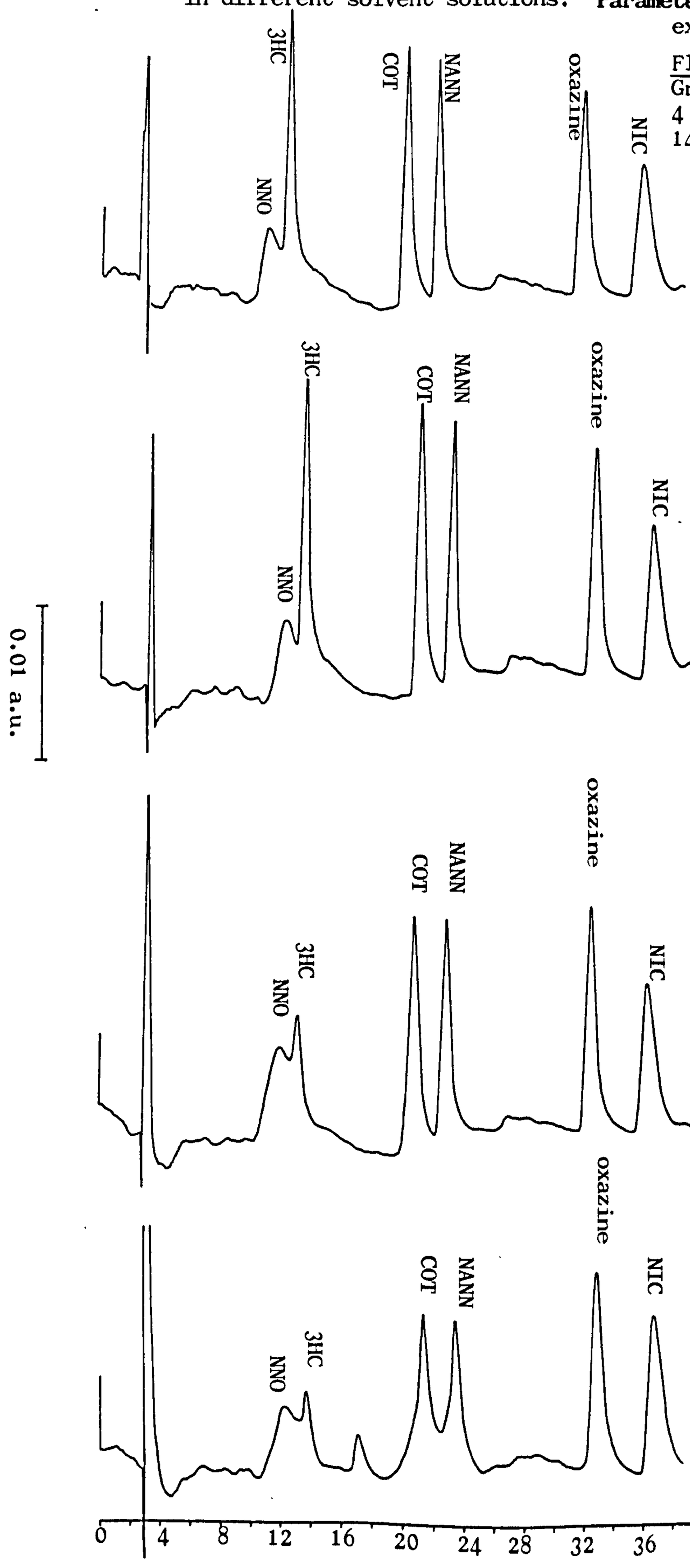
In all the extraction processes employed to date, the components of interest have been eluted from the Sep Pak using methanol, the extract being made up to its original volume with 0.2% phosphoric acid, pH adjusted to 7.25 with triethylamine, in a 50:50 ratio. A concentration step would be possible by simply injecting the extract in neat methanol or further concentration could be achieved by the removal of methanol by evaporation.

It was noted that when the standard mixtures were injected in 100% methanol, the peak shape and peak height seemed to deteriorate. This was investigated in detail using standard mixtures made up in 100% water, 75% water:25% methanol, 50% water:50% methanol, 25% water:75% methanol and finally 100% methanol. The resulting chromatograms are shown in figure 4.43. The effect is most striking for nicotine-1'-N-oxide and 3' hydroxycotinine, the two most polar components which are eluted early in the chromatogram. The peaks for the IS and nicotine are relatively unaffected.

These effects are in agreement with those noted by McCoy et al.[200] They replaced neat isopropanol with an isopropanol:water mixture to avoid peak distortion effects. The distortion effects are noted when the sample solvent has greater solvent strength than the mobile phase, as with methanol, the sample solvent and methanol/water, the mobile phase mixture. McCoy et al.[200] also reported that these effects were most severe for the earliest eluting components which was found to be the case in this investigation with nicotine-1'-N-oxide and 3' hydroxycotinine most affected.

Figure 4.43: Chromatograms of a standard mixture (10 μg ml^{-1}) injected in different solvent solutions. **Parameters:** see figure 4.2 except for:

Flow Rate: 1 ml min^{-1},
Gradient programme #6,
4 min initial hold at 14%B,
14% → 54%B over 20 mins



(a) Standard mixture in 100% H_2O

(b) Standard mixture in 75% H_2O: 25% MeOH

(c) Standard mixture in 25% H_2O:75% MeOH

(d) Standard mixture in 100% MeOH

4.8.6 Extraction of Smokers' Urine Samples

Extraction procedure 8, figure 4.20, can be used to clean up and extract the components of interest from a smoker's urine sample prior to HPLC analysis. Scheme 8 is a combination of extraction procedures 6 and 7, figures 4.12 and 4.18, where scheme 6 extracts nicotine, nicotine-1'-N-oxide and the IS and scheme 7 extracts cotinine and 3' hydroxycotinine.

In the first experiment to analyze a smoker's urine sample, smoker A, extraction procedures 6 and 7 were followed, the extracts from each being kept separately and analyzed separately.

The chromatogram showing the analysis of the extract from scheme 7 is presented in figure 4.44. The expected positions of 3' hydroxycotinine and cotinine have been marked and these positions were confirmed by spiking the extract with standard solutions (1000 $\mu g\ ml^{-1}$ in H_2O). In the smoker's extract, the peak corresponding to cotinine was very small, however that at the same retention time as 3' hydroxycotinine indicated a considerable quantity of this metabolite. Experiments carried out earlier on blank urine samples have shown that an interfering peak with the same retention time as 3' hydroxycotinine may be co-extracted, see sections 4.8.3 and 4.8.4. Therefore, the peak in the smoker's urine extract corresponding to the retention time of 3' hydroxycotinine may represent not only 3' hydroxycotinine but also the unidentified interfering compound.

A chromatogram showing smoker A's urine extract concentrated x 20 is presented in figure 4.45. The peak assigned to cotinine

Figure 4.44: Extraction Procedure 7 (figure 4.18), applied to Smoker A's urine sample (female)
Parameters: see figure 4.2 except for Flow Rate: 1 ml min^{-1} and Gradient Programme #6, 17% → 37%B over 15 mins

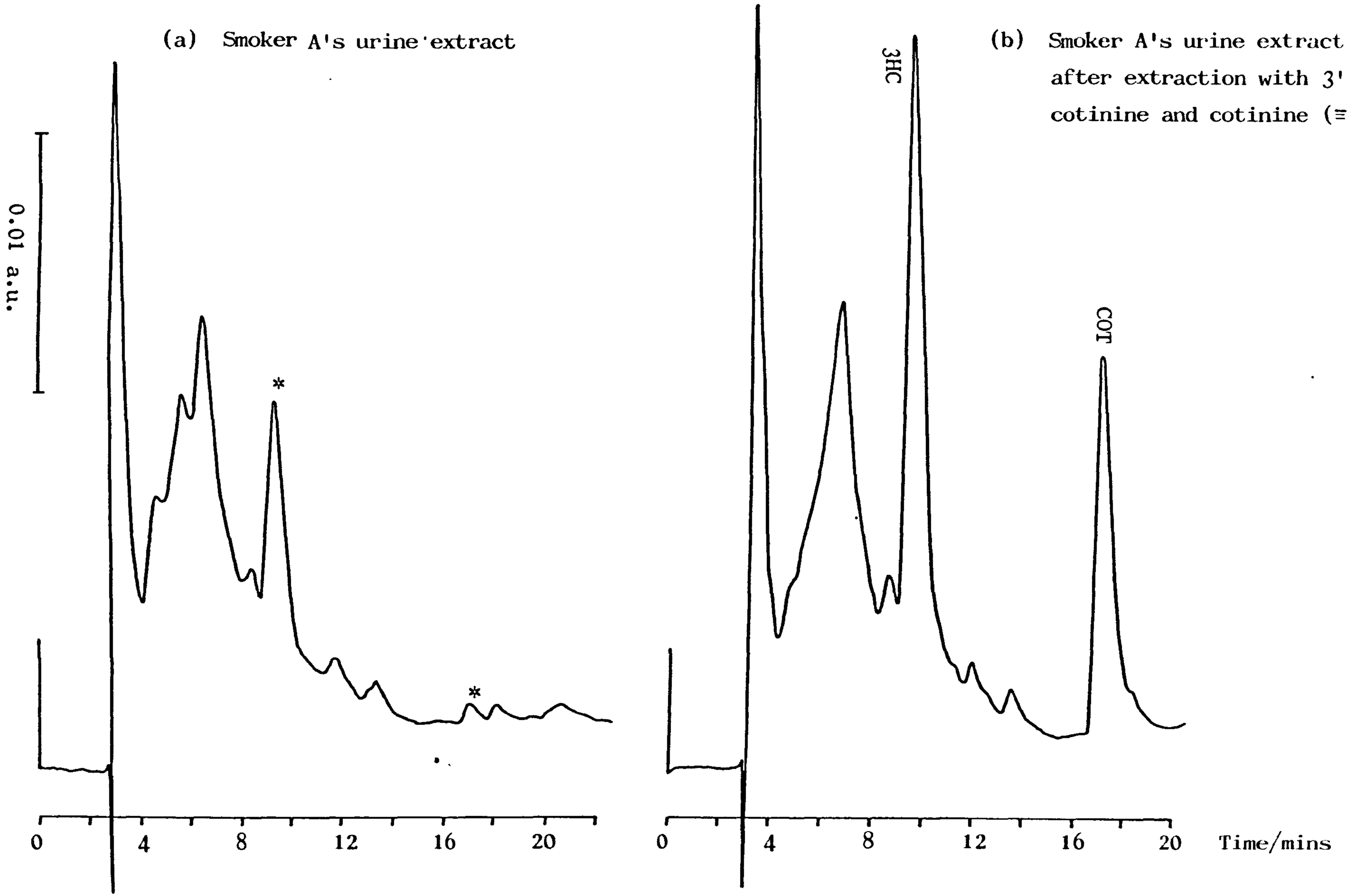
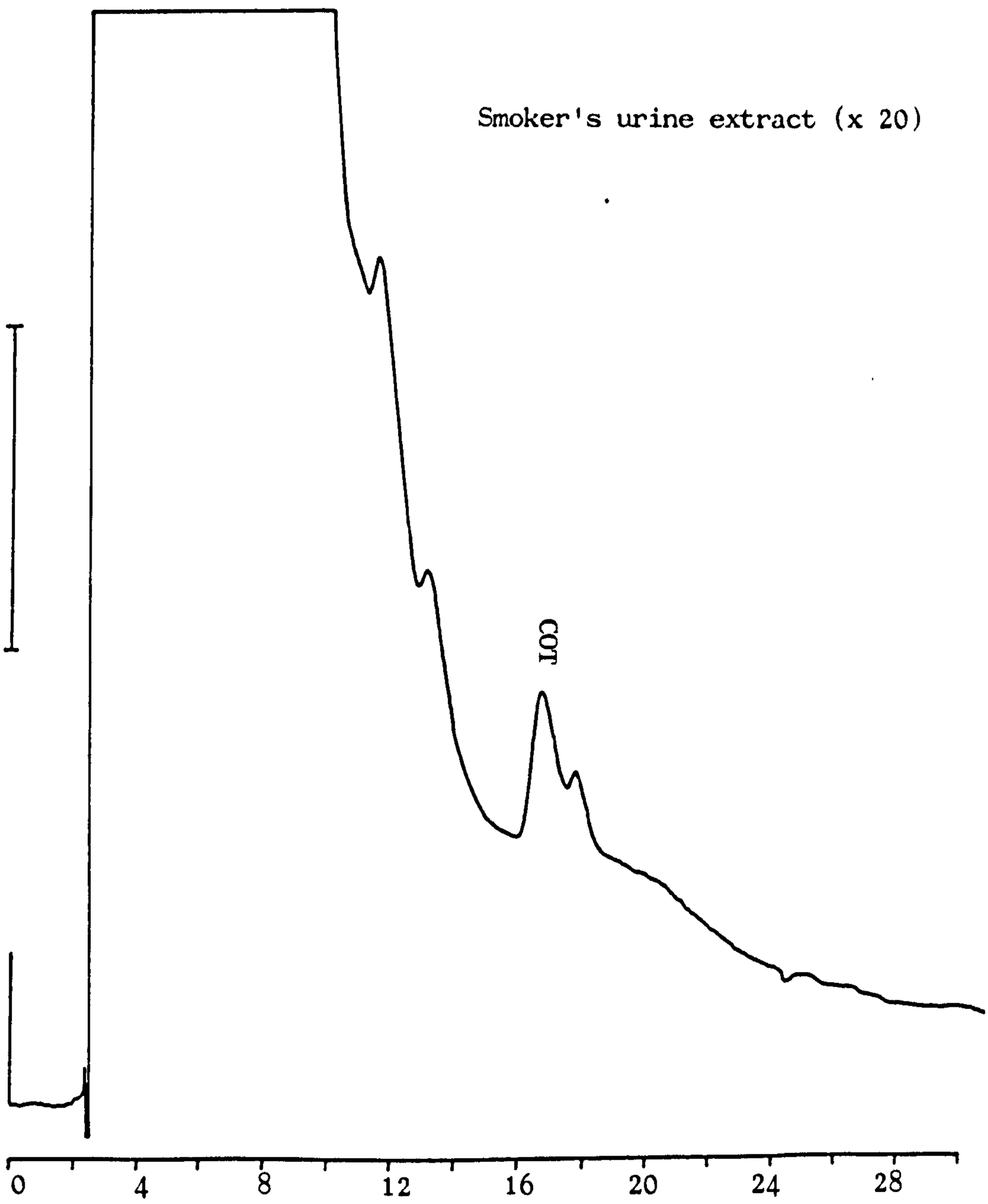


Figure 4.45: Extraction Procedure 7 (figure 4.18) applied to Smoker A's urine extract (female)

Parameters: see figure 4.44



was more readily distinguishable above the baseline noise, however the peak thought to be 3' hydroxycotinine was not visible owing to the many other co-extracted components in the extract.

Unfortunately the fraction extracted using scheme 7, figure 4.18, decomposed before analysis was possible, due to instrumental problems. Urine samples were usually extracted and analyzed on the same day.

The chromatogram in figure 4.46 shows the extract from smoker B's urine sample, extracted using procedure 8, figure 4.20. The clean-up was not very effective and due to the many components of no analytical interest present at the start of the chromatogram, 3' hydroxycotinine and nicotine-1'-N-oxide could not be identified. Peaks have been assigned to cotinine, IS and nicotine. Concentration of the extract x 2, as shown in figure 4.47, illustrated the difficulties already discussed; not only were the components of interest concentrated, the many other components present in the extract in high concentrations even after extraction were also concentrated, so making it impossible to identify nicotine or any of its metabolites.

Smoker B's urine sample was also analyzed using capillary GC with nitrogen sensitive detection.[206] The sample was extracted by extraction procedure 1, figure 4.1. The chromatogram showing the extract (x 8) is presented in figure 4.48. This analysis confirms the presence of nicotine and cotinine in the smoker's urine sample. The concentration of each of the components of interest in the smoker's urine has been quoted in brackets on the chromatogram.

Figure 4.46: Extraction Procedure 8 (figure 4.20) applied to Smoker B's urine sample (female). Parameters: see figure 4.36

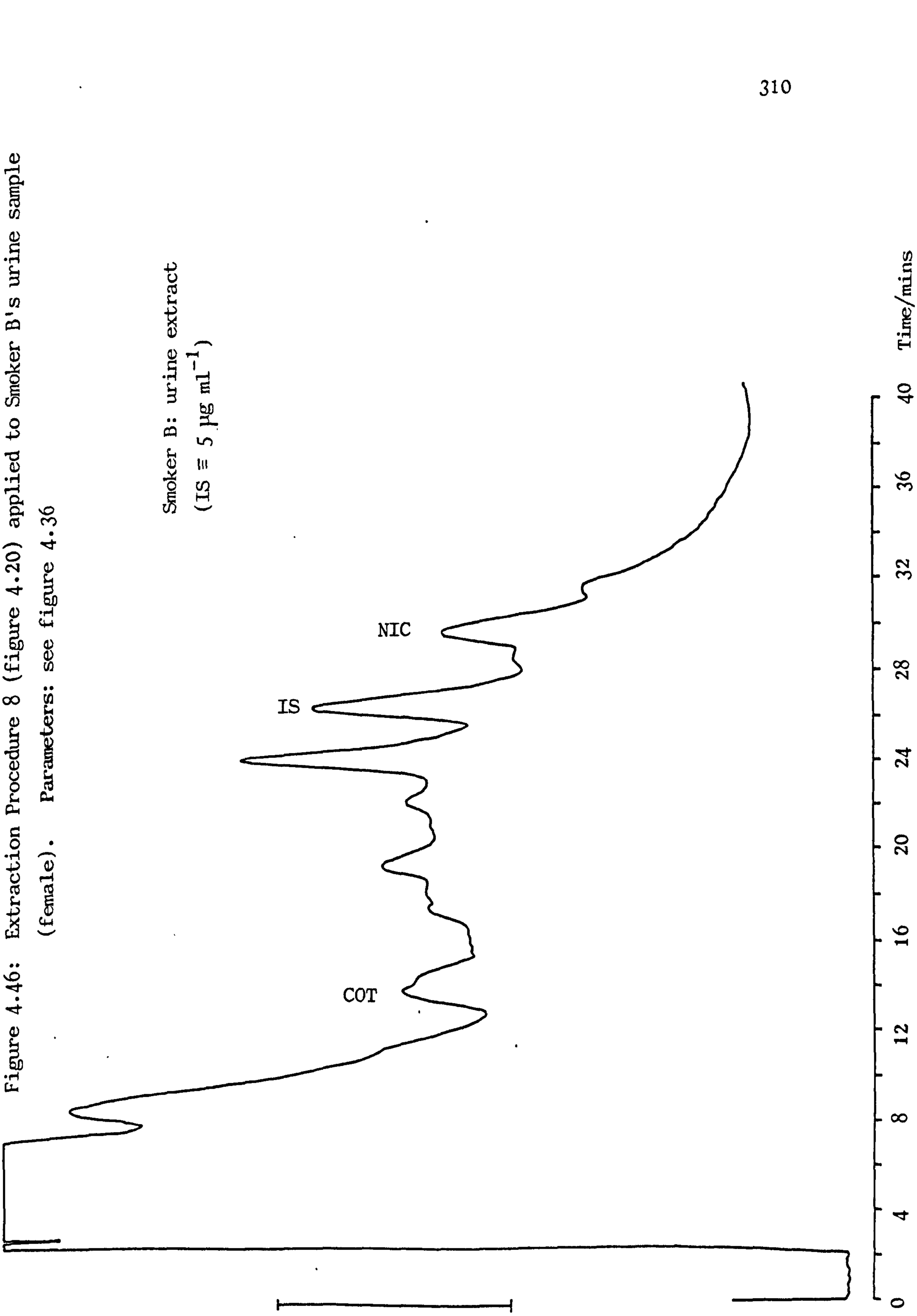
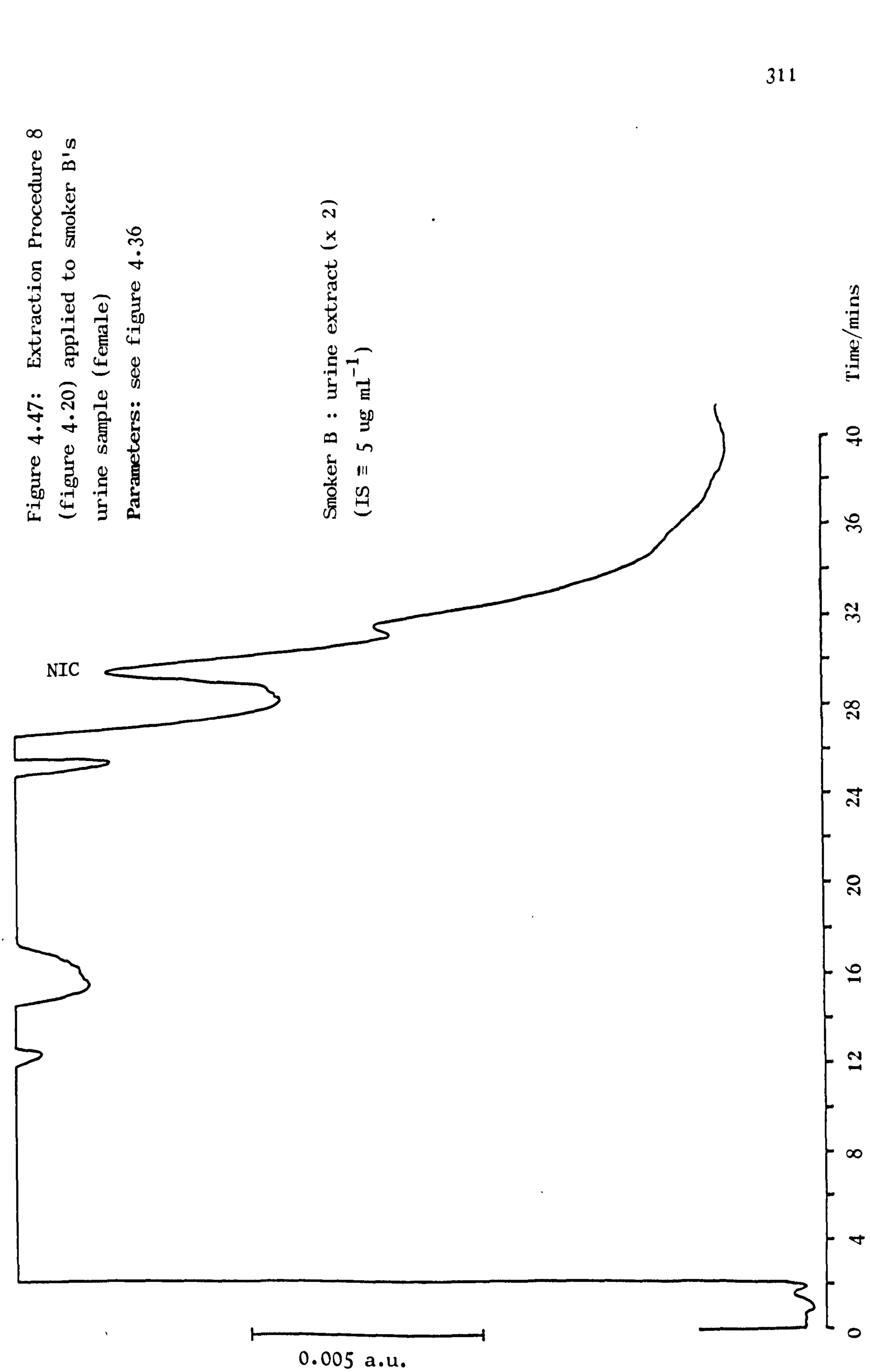


Figure 4.47: Extraction Procedure 8
(figure 4.20) applied to smoker B's
urine sample (female)
Parameters: see figure 4.36

Smoker B : urine extract (x 2)
(IS ≡ 5 ug ml^{-1})



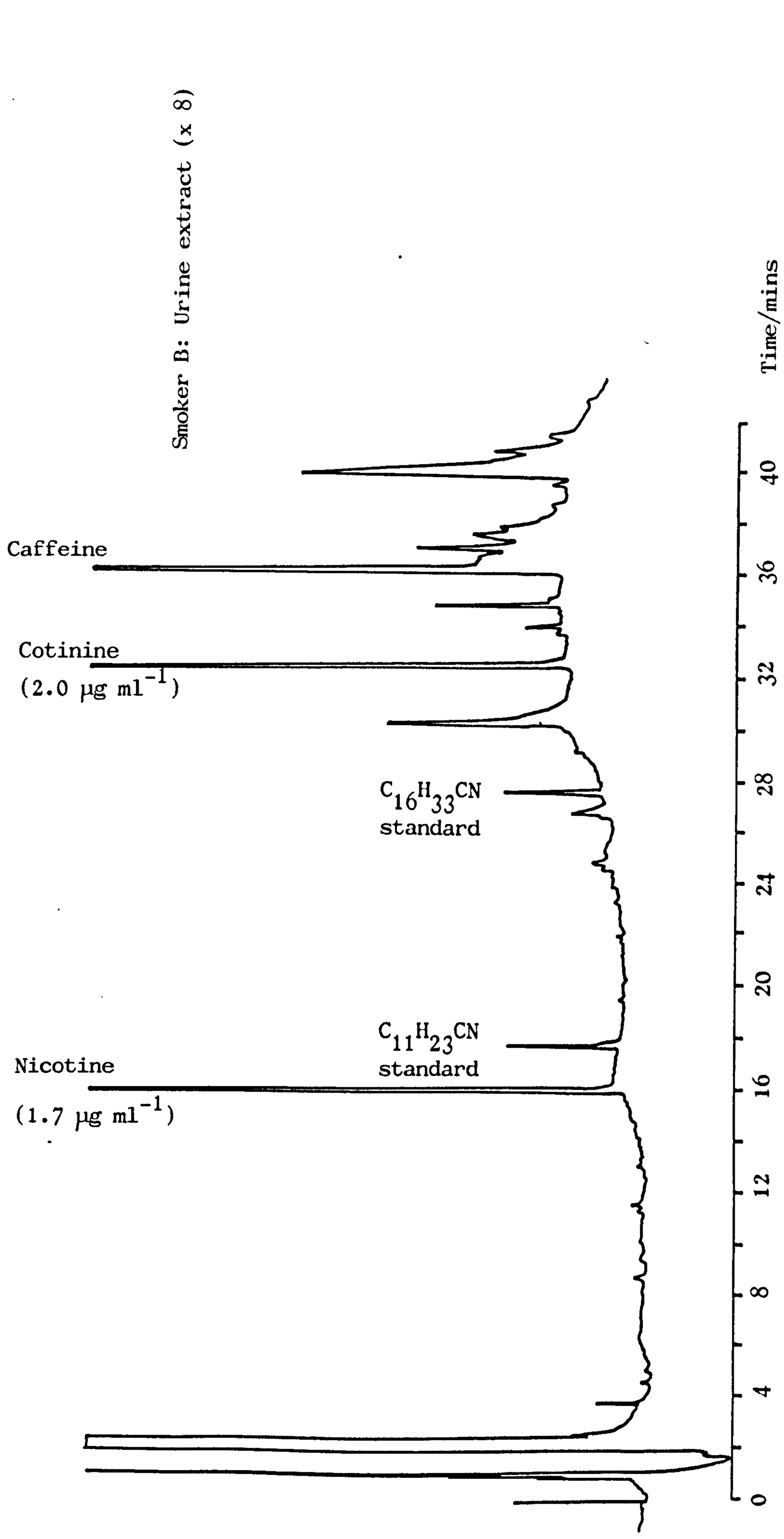


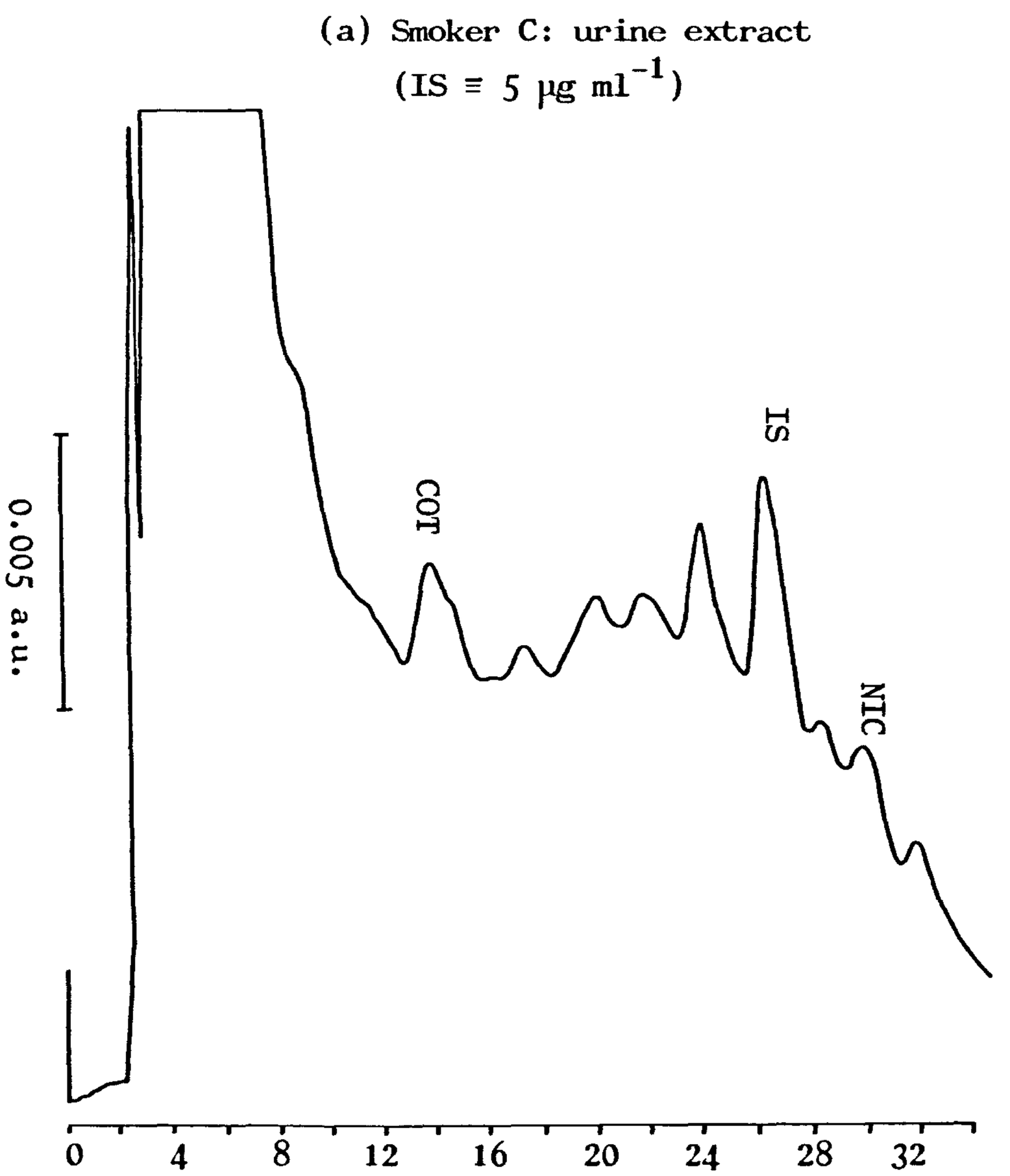
Figure 4.48: Chromatogram showing the capillary GC/AFID analysis of smoker B's urine sample extracted using procedure 1 (figure 4.1).

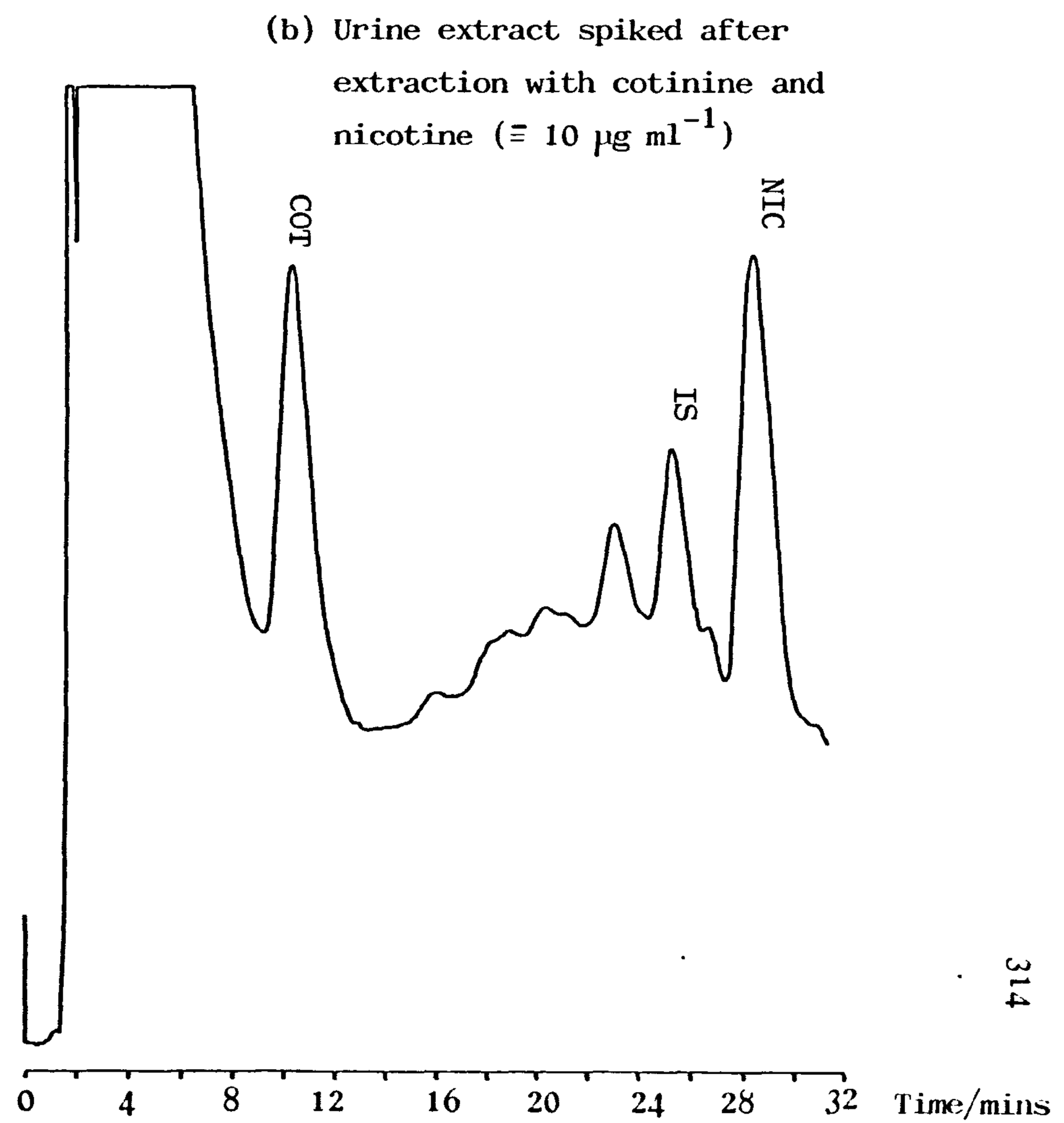
Extraction of another smoker's urine sample, smoker C, by extraction procedure 8, figure 4.20, gave a similar result to that obtained for smoker B. It was possible to assign peaks to cotinine, IS and nicotine as shown in figure 4.49. Again peak assignment was confirmed by spiking the extract with the corresponding standard solutions (1000 $\mu g\ ml^{-1}$ in H_2O). The clean-up of the sample was not sufficiently good for the earliest eluting components of interest, nicotine-1'-N-oxide and 3' hydroxycotinine, to be identified. Confirmation of the presence of nicotine and cotinine was available from GC analysis of the urine extract, shown in figure 4.50, the corresponding concentrations of nicotine and cotinine in the smoker's sample being quoted in brackets on the chromatogram.[206]

One other smoker's urine sample has been included; smoker D's sample was extracted by procedure 9, figure 4.32. A relatively clean extract was obtained as shown in figure 4.51; however, without the use of a concentration step it was very difficult to distinguish nicotine or cotinine from the baseline noise. A large peak was present at the retention time corresponding to 3' hydroxycotinine/ nicotine-1'-N-oxide. As in the case of smoker A, figure 4.44, although some 3' hydroxycotinine/nicotine-1'-N-oxide may have been present, there was almost certainly another component, also present, which caused an interference.

The identification of nicotine and cotinine is illustrated in figure 4.52, where the extract, concentrated x 5, was spiked with standard solutions (1000 $\mu g\ ml^{-1}$ in H_2O). The concentration and spiking of the extract, figure 4.52, revealed that the peak, originally thought to be cotinine, was in fact made up of more

Figure 4.49: Extraction Procedure 8 (figure 4.20) applied to smoker C's urine sample (female)
Parameters: see figure 4.36





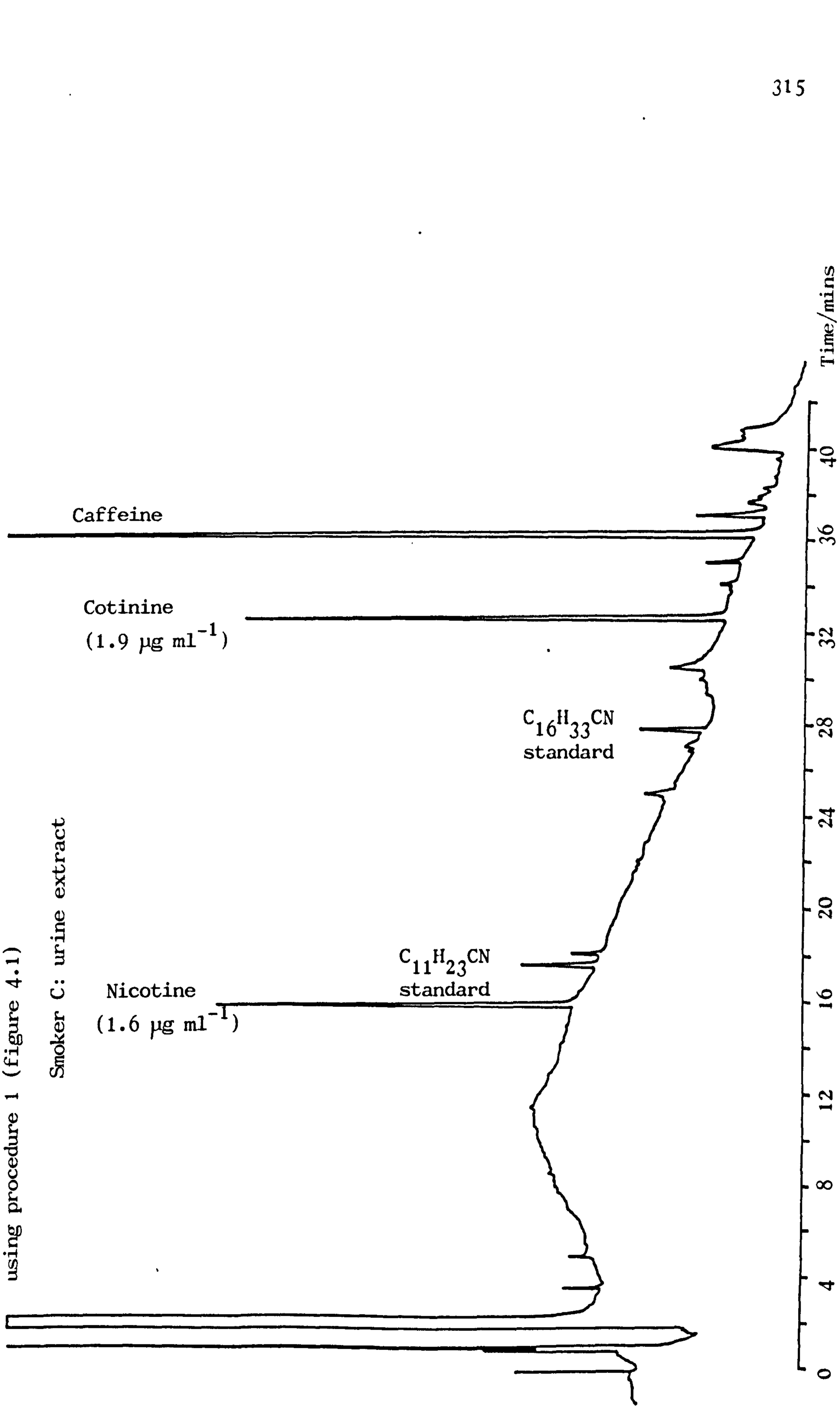


Figure 4.50: Chromatogram showing the capillary GC/AFID analysis of smoker C's urine sample (female) using procedure 1 (figure 4.1)

Smoker C: urine extract

Figure 4.51: Extraction Procedure 9 (figure 4.32) applied to smoker D's urine sample (female) **Parameters:** see figure 4.36

Smoker D: urine extract

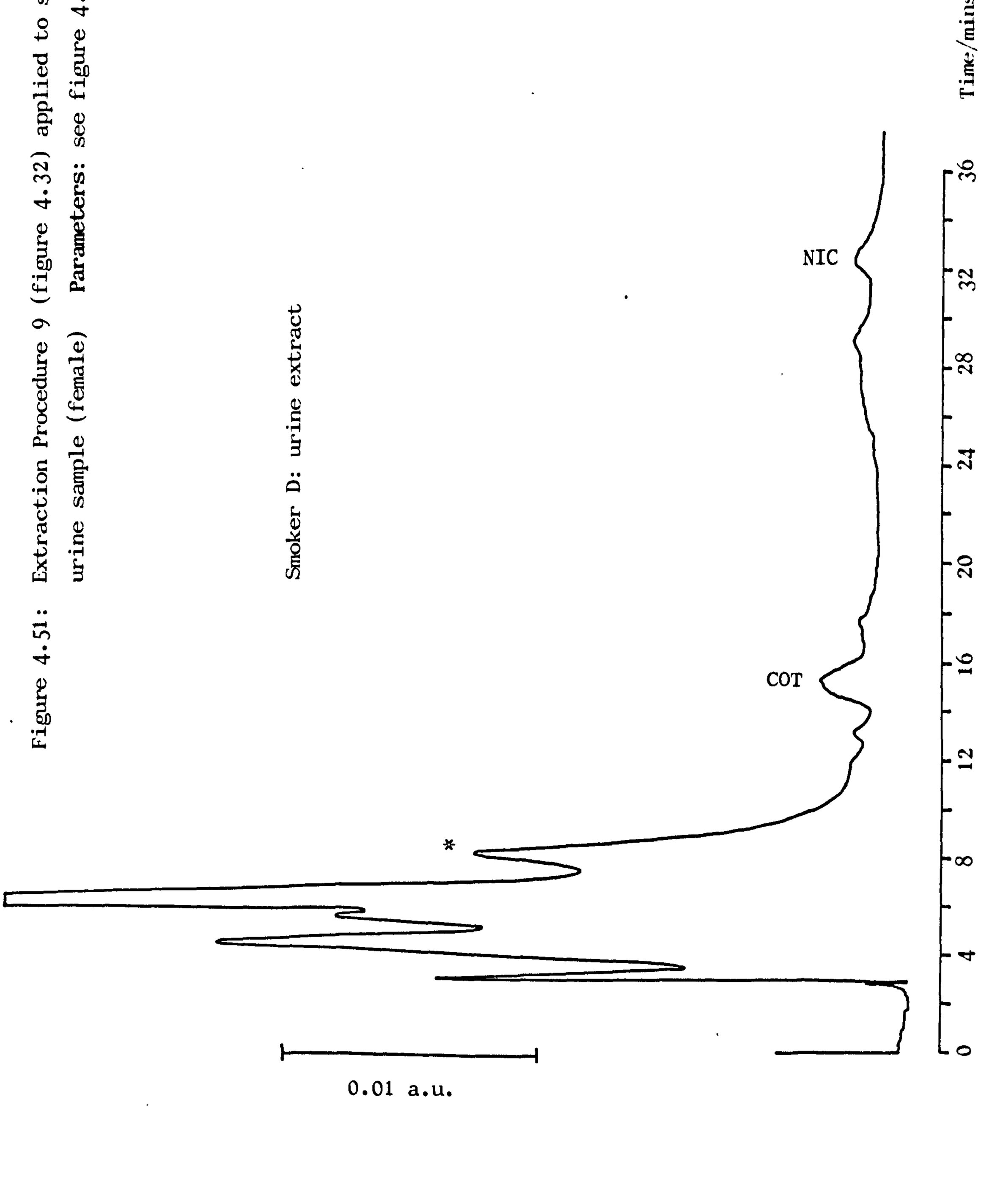
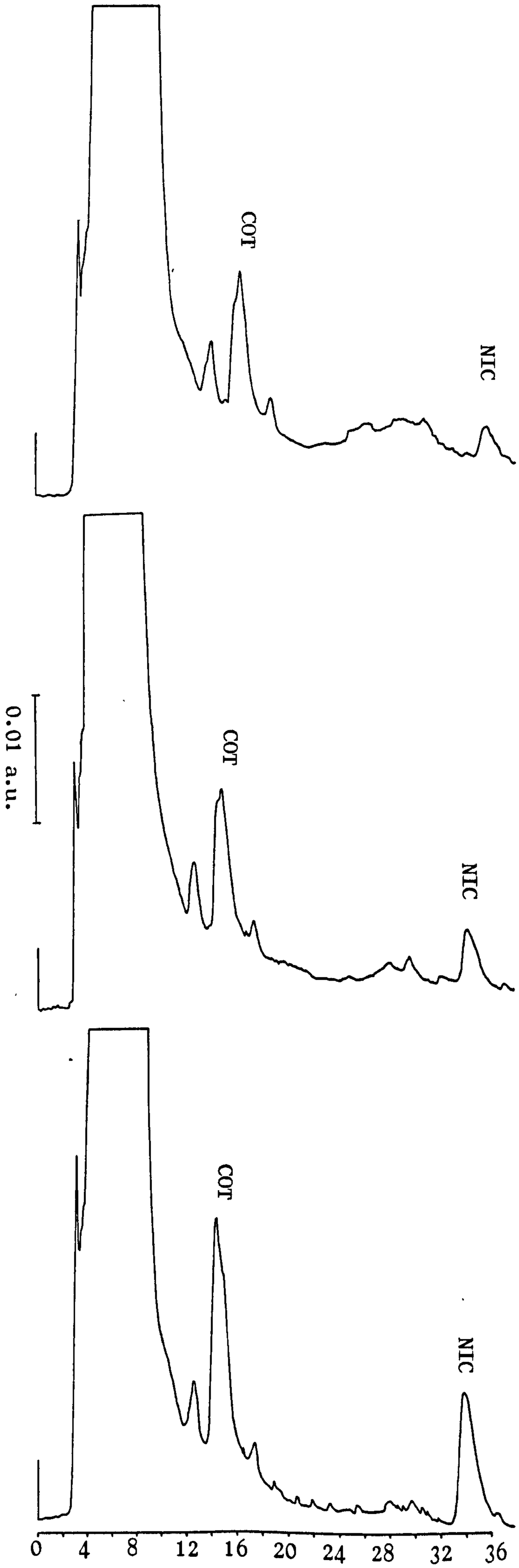


Figure 4.52:
Extraction Procedure 9 (figure 4.32) applied to smoker D's urine sample (female)

Paramaters: see figure 4.36



(a) Smoker D:
urine extract (x 5)

(b) Urine extract (x 5) spiked with cotinine and nicotine

(c) Urine extract (x 5) spiked with more cotinine and nicotine

than one component, cotinine being eluted as the first of these. The presence of nicotine and cotinine was again verified by GC analysis, see figure 4.53.[206]

Analyses of the smokers' urine samples has underlined the necessity for a concentration step and therefore the need for an effective clean-up procedure. The concentration of nicotine and its metabolites, cotinine, 3' hydroxycotinine and nicotine-1'-N-oxide, in the samples were very low. More efficient chromatography and better resolution are also required due to the numerous co-extracted components in the extracts.

It was not possible to make a quantitative determination of nicotine and its metabolites present in the smokers' urine samples - it was difficult to identify the peaks corresponding to the components of interest and in several examples even this was not possible for nicotine-1'-N-oxide and 3' hydroxycotinine.

Confirmation of the presence of nicotine and cotinine was available from analyses of the smokers' urine samples by GC. The GC analysis referred to employed capillary GC with a nitrogen sensitive alkali tip detector which provide efficient GC and an enhanced response to compounds containing nitrogen, so reducing the importance of the clean-up/extraction procedure required. The GC analysis confirmed that nicotine and cotinine were present in the smokers' urine samples. The peaks in the extract analyzed by HPLC were identified by spiking the extract with the standard solutions (1000 $\mu g\ ml^{-1}$ in H_2O). To verify peak identification LC-MS or LC-FTIR would be required.

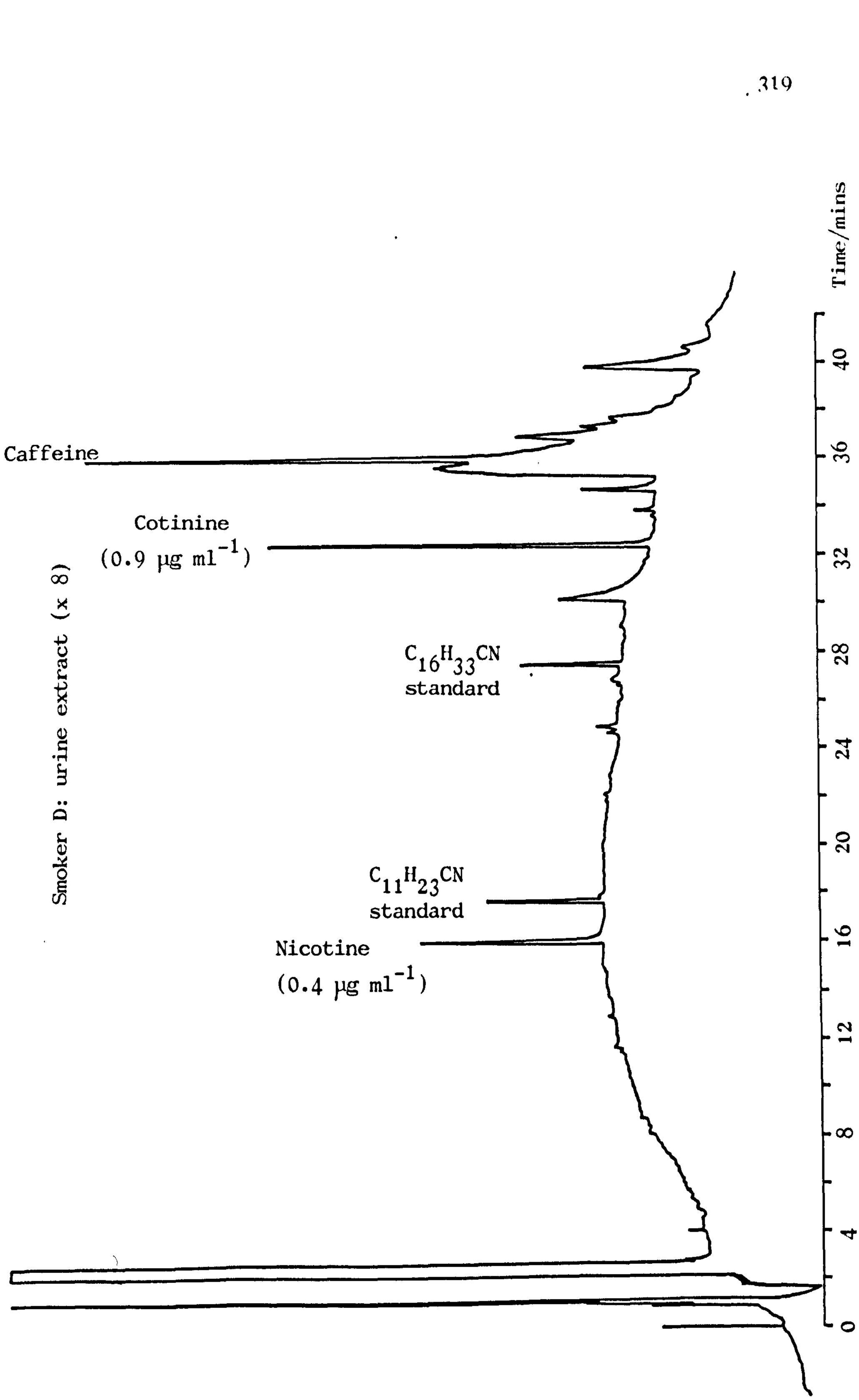


Figure 4.53: Chromatogram showing the capillary GC/AFID analysis of smoker D's urine extract (female) extracted using procedure 1 (figure 4.1)

CHAPTER 5

EXPERIMENTAL

5.1 Instrumentation

5.1.1 Isocratic HPLC

Pump : Shimadzu LC-5A Liquid Chromatograph

Injection valves : Rheodyne Model 7010

Loop capacity : 20 µl

Detector : Pye Unicam LC-UV variable wavelength detector

Chart Recorders : Servoscribe 1s and Kipp & Zonen BD8 Multirange

10 mV f.s.d.

Chart speed 5 mm min^{-1}

Integrator : Hewlett Packard 3390A Integrator

The preliminary chromatographic trials were carried out under isocratic conditions. The operating conditions have been reported on the individual chromatograms included.

5.1.2 Gradient Elution

Instrumentation as in section 5.1.1, except:

Pumps : Waters Associates Model 6000A solvent delivery system (2)

Gradient programmer : Waters Associates Model 660 Solvent Programmer

Loop capacities : 50 µl and 100 µl

Operating conditions have been reported on the individual chromatograms included.

5.2 Solvents and Reagents

Nicotine and its metabolites, nicotine-1'-N-oxide, cotinine and 3' hydroxycotinine, together with the following compounds which were tested as internal/chromatographic standards, β-nicotyrine, N-ethyl nornicotine, methyl-4-(3-pyridyl)-4-oxobutyrate and 2-methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine, were kindly donated by Carreras Rothmans Research Division. ± Anabasine was supplied by the Sigma Chemical Co., Poole, Dorset, and caffeine was obtained from BDH Biochemicals, Poole, Dorset.

Methanol (GPR grade) was supplied by Wilcot Industrial, Bristol, Avon, and was single distilled into glass before use. Water was single distilled into polythene before use. Acetonitrile (HPLC grade) was obtained from Fisons, Loughborough, Leicestershire and was used as received. Sodium acetate trihydrate (AnalaR grade), triethylamine (GPR grade), diethylamine (GPR grade), orthophosphoric acid (AnalaR grade), glacial acetic acid (AnalaR grade) and mobile phase additives, n-heptanol (GPR grade), n-octanol and nitromethane were all supplied by BDH Chemicals Ltd., Poole, Dorset. Pentadeca-fluoro-octanoic acid was obtained from the Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Sep Paks (C_{18} type) were supplied by Waters Associates, Northwich, Cheshire. Concentrated hydrochloric acid (AnalaR grade), sodium hydroxide (pellets, AnalaR grade) and dichloromethane (AnalaR grade) were all supplied by BDH Chemicals Ltd., Poole, Dorset. The ion-pairing agents dodecylsodium sulphate and sodium 1-heptane-sulphonate anhydrous were obtained from Lancaster Synthesis Ltd., Morecambe.

Helium gas used for degassing of the mobile phase was supplied by BOC (Special Gases), Bristol, Avon.

Smokers' urine samples were obtained from the Bristol Maternity Hospital.

5.3 Columns

The packing materials used in the course of this work have been listed in tables 2.2, 2.3 and 2.9, together with the manufacturers' specifications and column dimensions.

All columns were packed in the laboratory. A slurry of the packing material in a supporting balanced-density solvent ($\sim$ 1 g of packing material to 10 ml of solvent) was prepared. Methanol containing cyclohexanol (3 drops per 10 ml methanol) was employed as the supporting liquid. The mixture was homogenized and degassed using ultrasonic vibration for $\sim$ 15 mins. The slurry was then placed in a reservoir over the column to be packed, which had been filled with a suitable solvent, e.g. propan-2-ol, and was pumped into the column using a N_2-driven constant pressure hydraulic pump (Haskel MCP-71) set at 6500 p.s.i.

The Resolve C_{18} 5 μ Radial PAK cartridges were supplied by Waters Associates.

5.4 Standard Solution Preparation

Stock solutions (1000 μg ml^{-1}) in distilled water were prepared for nicotine, cotinine, nicotine-1'-N-oxide, 3' hydroxycotinine, anabasine and the substances tested as internal standards. Working standards were obtained by serial dilution with distilled water. All stock and standard solutions were stored in the refrigerator at ∿ 4°C until required.

5.5 Mobile Phase Preparation

5.5.1 For Ion Chromatography

Sodium acetate (0.3M) was mixed with methanol in a 1 L container to give the desired ratio (70:30) and the final pH was adjusted to 4.5 with glacial acetic acid.

The molarity of the sodium acetate, the pH of the final solution and the percentage of methanol in the mobile phase were varied in an attempt to effect a separation.

5.5.2 For RP-IIC

The ion-pairing agent PDFOA was added to methanol in the desired concentration, 1000 μg ml^{-1}. A sodium acetate solution (0.3M) and the solution of PDFOA in methanol were mixed in the desired ratio (70:30) and the pH adjusted to 4.5 with glacial acetic acid. Anti-tailing agents, triethylamine or diethylamine, were added, in some cases, as 0.1% of the final mixture. The concentration of PDFOA, the pH of the buffer solution and the ratio of buffer

to methanol solution were varied in the course of the experiments and have been reported on the individual chromatograms included.

5.5.3 For Reverse Phase Partition Chromatography

Orthophosphoric acid, 0.2% in distilled water, was prepared and the pH adjusted to 7.25 with triethylamine. This solution was mixed with methanol in the desired ratio, as stated on the individual chromatograms included.

The introduction of the gradient system, which consisted of two pumps under the control of a gradient programmer, removed the need for premixing the solutions in the desired ratio, as the ratio was set by the gradient programmer.

5.5.4 General Procedure

All eluents were degassed under a gentle stream of He for approximately 20 minutes before delivery. Degassing was continuous throughout the course of the experiments in order to prevent gases dissolving in the mobile phase.

5.6 Internal Standard

As already reported in section 5.4, all possible IS solutions were prepared (1000 $\mu g\ ml^{-1}$) in distilled water. In experiments to study their retention characteristics under different conditions and also their extraction from standard solutions in distilled water, working standards were required and these were prepared

by serial dilution with distilled water.

Internal standards were added to urine samples before extraction as a concentrated 'spike', e.g. 200 µl volume of IS (1000 µg ml^{-1} solution in distilled water) was added to a 20 ml urine sample i.e. = 10 µg ml^{-1}.

If a concentration step was to be included in the extraction procedure, the level of IS, added to the sample, was adjusted accordingly.

5.7 Clean-up/Extraction

In several experiments pre-packed solid-phase extraction cartridges (Sep Pak, C_{18} type) were used in the clean-up/extraction procedure. All Sep Pak C_{18} cartridges were activated, before use, by flushing with methanol (2 ml) followed by distilled water (2 ml) at a flow rate of ~ 2 ml min^{-1}.

A minority of urine samples required centrifugation prior to clean-up/extraction. This was carried out using the BTL bench centrifuge set at 2500 r.p.m. for 10 mins.

Samples to be cleaned up/extracted by means of a Sep Pak were passed through the Sep Pak slowly, flow rates of 2-3 ml min^{-1} being used.

5.8 Gradient System - general procedure

At the start of each working day, a blank gradient run, one where no injection of solvent or sample was made, was examined to ensure that nothing was eluted from the column as the eluent strength increased over the desired programme range. This procedure was repeated at intervals over the course of the day's work.

If any unidentified peaks were encountered the column was flushed with the solvent of highest eluent strength for $\sim \frac{1}{2}$ hr. After a period of equilibration at the desired starting eluent composition, a repeat blank gradient was run. This process was repeated until any components which had been strongly retained by the packing material, and were likely to cause an interference in subsequent analysis, had been removed.

During the analysis of urine samples, blank gradient runs were examined more frequently.

The gradient programmes used for the analyses have been reported on the individual chromatograms included. The gradient programmes employed were optimized during the lifetime of a particular column/ cartridge, to achieve the best possible separation of the components of interest.

5.9 Maintenance of the HPLC Equipment

Routine maintenance of equipment included regular cleaning of the reservoir filters and the UV flow cell and was achieved by ultrasonic vibration in a suitable solvent.

Solvent delivery systems, especially those pumping buffer solutions, were flushed with methanol when not in use for periods of 1 week or longer.

CHAPTER 6

DISCUSSION, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 HPLC and the Analysis of Nicotine and its Metabolites

GC has been the dominant technique in the determination of nicotine and its in vivo metabolites. Using this technique it is possible to determine only the volatile, thermally stable components. Although quantitative analysis of nicotine and cotinine has been accomplished, determination of nicotine-1'-N-oxide, which is not thermally stable at the temperatures required for GC analysis, is only possible by reducing it to nicotine and noting the increase in nicotine present in a subsequent determination.[82]

HPLC was an obvious alternative choice for the determination of nicotine and its metabolites, being carried out at room temperature. The analysis of the components of interest by a single injection was thought possible, given the two competing phases (stationary and mobile) in HPLC which can be used to effect separations not possible by GC with just one (stationary phase).

UV detection was the only possible mode of detection readily available and although there were doubts about its sensitivity, it was thought that this problem could be overcome by concentration of the urine extract.

Several modes of HPLC were investigated: ion-chromatography, reverse-phase ion-interaction chromatography and reverse phase partition chromatography.

Using both ion chromatography and RP-IIC, separation of the three components of interest, nicotine, cotinine and nicotine-1'-N-oxide, proved to be impossible. Alterations in the pH of the mobile phase, the percentage of MeOH in the mobile phase, etc., were tried

without success and hence these modes of chromatography were abandoned. Reverse phase partition chromatography, however, immediately looked more promising. A separation of all three components of interest was achieved on a μ-Bondapak column with a mobile phase of 0.2% H_3PO_4, pH adjusted to 7.25 : MeOH (80:20).

6.2 Reverse Phase Partition Chromatography

In reverse phase chromatography, the stationary phase is hydrophobic, hence polar substances are eluted first. The lower the polarity of the mobile phase the higher is its eluent strength and hence the shorter the retention times of the components. This was found to be the case for nicotine, cotinine and nicotine-1'-N-oxide. As the percentage of the organic modifier in the mobile phase decreased and hence the lower its eluent strength, retention increased. For nicotine, which was strongly retained by the ODS packing material, retention increased to the point where the nicotine peak was broad and tailing.

Many different ODS phases were investigated together with Hypersil phenyl and Nucleosil NO_2, in an attempt to effect a separation of the three components of interest, in a reasonable analysis time (e.g. < 30 mins) and with a k' value for nicotine-1'-N-oxide, or the first peak of interest eluted from the column, > 2.0.

The ODS packing materials, although they all have the C_{18} functional group bonded to the silica, exhibit differences. Hydrophobicity, the carbon loading, surface area, pore size, particle

size and end-capping are major factors which differentiate reverse phase packing.

C_{18} reverse phase packings are produced by chemically bonding a C_{18} hydrocarbon to a silica support. Inevitably some of the silanol (Si-OH) groups remain unreacted and these can have an effect on the separation. Therefore the separation mechanism may not be purely partition but adsorption may also play a part. To minimize the effect of unreacted silanol groups an 'end-capping' step can be included after the C_{18} hydrocarbon has been chemically bonded to the support. The effect of the silanol groups is not necessarily related to the number present but to their accessibility. Hence there are differences even among packing materials which have been 'end-capped'.

Of all the bonded phases investigated the Resolve C_{18} 5 μ packing material was the most promising when the retention of nicotine-1'-N-oxide was considered. However, it was impossible to consider the use of this column without the use of gradient elution as both cotinine and nicotine were strongly retained by this packing material when the eluent strength was such that the k' for nicotine-1'-N-oxide was $\sim$ 2.

The Resolve C_{18} 5 μ packing material was available in the Waters flexible walled cartridges. The use of flexible walled cartridges and radial compression results in higher efficiency due to the uniform packing structure throughout the column and hence the elimination of the so called "wall effects". Improved chromatography and an 8 mm ID for the Radial PAK cartridge made a switch to a 100 μl loop possible, so increasing sensitivity without

the need for a high sensitivity setting on the detector.

The Resolve C_{18} 5 μ cartridge proved to be very successful. An excellent separation of nicotine-1'-N-oxide, cotinine and nicotine was achieved and, later, 3' hydroxycotinine was also included in the analysis and separated from the other components of interest, although baseline resolution was generally not achieved.

Two excellent chromatographic standards were examined, N' acetyl nornicotine (k' = 4.18) and 2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxazine (k' = 7.20). 2 methyl-6-(3-pyridyl)-tetrahydro-(1,2)-oxaxine was used as an IS as it could be taken through the clean-up/extraction procedures chosen for the analysis of smokers' urine samples. N' acetyl nornicotine could not be used as an IS and hence it was discarded.

Further improvements in the chromatographic efficiency would be possible by reducing all column fittings and connecting tubing to a minimum, thermostatting the column and perhaps the use of a multi-step gradient system.

However, the chromatographic method of analysis developed was considered sufficiently good to warrant statistical analysis of the instrument response and the development of a clean-up/extraction procedure, leading to a trial on smokers' urine samples.

6.3 Statistical Analysis

Statistical analysis of the instrument response in the analysis of nicotine and its metabolites in standard solutions was carried out. A comparison of the statistical data calculated for each analyte peak was made using peak height, peak height ratio, peak area and peak area ratio measurement parameters.

The within-run precision, repeatability, was measured as the RSD (%) for each analyte and by the method of quantitation. The mean RSD for all analyte peaks by different methods of quantitation ranged from 2.9% for the peak height measurement parameter to 6.3% for the peak area measurement parameter.

The between-run precision, reproducibility, expressed as the mean RSD for all analyte peaks over all concentrations by different methods of quantitation, ranged from 4.9% for the peak height ratio measurement parameter to 7.0% for the peak area measurement parameter.

The best within-run precision was a result of quantitation by peak height measurement. When between-run precision was measured, the inclusion of an IS, which showed an improvement in the mean RSD averaged over all concentrations and standards for each method of quantitation, resulted in peak height ratio (RSD = 4.9%) followed by peak area ratio (RSD = 6.7%) being the best methods of quantitation.

Peak area measurements were expected to be superior to peak height measurements especially as gradient elution was involved, peak height measurements being more susceptible to variations in the chromatographic conditions. However, due to the use of the

gradient which gave a fluctuating baseline, the integrator was inconsistent in marking the start and end-points of the peaks. This erratic integration of peaks contributed to the low precision values reported when peak area measurements were used.

Bakalyar and Henry[207] reported that in gradient elution if flow control is good but solvent composition cannot be maintained precisely, peak area measurements yield better quantitative results because area is relatively independent of composition. However, if flow control is poor but composition can be maintained precisely, peak height measurements yield better quantitative results because height is relatively independent of flow rate.

In addition, McCoy et al.[200] reported that when peaks are poorly resolved from the solvent front or an earlier eluting peak, or if the baseline is not well established, peak height measurements appear to provide more precise quantitation. This work was carried out using isocratic elution. In general high accuracy is sacrificed when peaks are fused. Aiken et al.[208] stated the need for baseline resolution of components, to provide good quantitative analysis.

Therefore, the higher precision obtained when peak height is the measurement parameter may be due to the lack of baseline resolution for some components, in particular nicotine-1'-N-oxide and 3' hydroxycotinine, and the fact that composition can be maintained precisely (common with older liquid chromatography pumping systems) but flow control is poor.

The inclusion of the IS resulted in an improvement in the precision of the experiment, when the mean RSDs for all analyte peaks over all concentrations by different methods of quantitation

were examined, the exception being the within-run precision when peak height was the measurement parameter.

Although the inclusion of an IS/chromatographic standard can result in an improvement in the precision of the method, the internal standard also monitors analyte losses during the extraction procedure. This is extremely important, as the variability in analyte losses during extraction can contribute to very low precision values.

The lack of precision for individual components by particular methods of quantitation are apparent from the graphs showing the calibration data together with the Working-Hotelling 95% confidence region and the 95% confidence bounds for 90% of future observations. The high LOD values calculated were higher than expected in some instances, again a reflection of the poor precision achieved. Although the levels of nicotine and its metabolites in smokers' urine samples were expected to be low it was still considered that it would be possible to measure the levels provided an effective clean-up/extraction was developed, a concentration of the extract was incorporated in the clean-up/extraction scheme and analyte losses during the scheme were negligible.

Ideally, calibration curves over the expected concentration range should be constructed for urine samples which have known concentrations of nicotine and its metabolites and have been processed through the whole extraction procedure. The precision values obtained for the quantitation of nicotine and its metabolites in standard solutions would undoubtedly be better than those possible for the components in urine extracts due to the variability in analyte loss during extraction. Inclusion of an IS is critical

for this reason as it should monitor analyte losses during extraction and hence prevent a deterioration in the overall precision of the method.

6.4 Urine Sample Analysis

The clean-up/extraction of urine samples proved to be extremely difficult. The main reason was the wide ranging polarities of nicotine and its metabolites, hence attempts to extract them resulted in many components of no analytical interest being co-extracted.

From the smokers' urine extracts chromatographed it was obvious that the clean-up was not satisfactory. The quantitation of early eluting peaks, namely nicotine-1'-N-oxide and 3' hydroxycotinine, was impossible.

A successful application of the chromatographic method developed required a very clean extract. The levels of nicotine and its metabolites, expected to be low even in smokers' urine samples, made a concentration step necessary, hence a clean extract was critical.

Many GC methods of analysis have employed nitrogen sensitive alkali tip detectors, which reduce the requirements for a very stringent clean-up process. Unlike AFID, UV detection was not very selective or very sensitive. The use of capillary GC also made efficient chromatography with high resolution possible.

Peak identification was aided by spiking the extract with

the corresponding standard solution (1000 μg ml^{-1}) in H_2O. Other techniques such as LC-FTIR or LC-MS would confirm the peak identity and these techniques may be required if this method is to be used for quantitative analysis of nicotine and its metabolites. Unfortunately, these modes of detection were not available during the course of this work.

6.5 Conclusions and Suggestions for Further Work

From the experimental results reported in this study, it must be concluded that this HPLC method is not suitable for the quantitative analysis of nicotine and its metabolites in smokers' urine samples.

The concentrations of nicotine and the metabolites in smokers' urine samples were found to be low (< 3 μg ml^{-1}) and the analyses of these samples confirmed the necessity for a concentration step and therefore a very effective clean-up procedure. Although many of the extraction procedures were successful in extracting the components of interest, the clean-up was not always satisfactory. Many components of no analytical interest were co-extracted and were eluted from the column with k' values in the range 0-3. Frequently, the earliest eluting peaks of interest, nicotine-1'-N-oxide and 3' hydroxycotinine, could not be identified due to these interfering components.

Taking into consideration the low levels of nicotine and its metabolites present in smokers' urine samples and the difficulty

in extracting these components with widely different polarities, UV detection was not sensitive or selective enough to enable quantitative analysis to be carried out.

Further work, using the method of analysis developed in this study, requires a different method of detection, one which is more sensitive and more selective, therefore making the extraction procedure less critical. The advantages of GC analysis with nitrogen sensitive detection have been shown.

Pre- or post-column derivatization may also make an HPLC quantitative method of analysis for nicotine and its metabolites viable, as this may also provide the increased sensitivity needed and the possibility of only a simple clean-up procedure being required.

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